

SERUM HEPCIDIN LEVELS IN NORMAL PREGNANCY

DISSERTATION

Submitted to

THE TAMILNADU DR MGR MEDICAL UNIVERSITY

In partial fulfillment for the degree

DOCTOR OF MEDICINE

IN

BIOCHEMISTRY- BRANCH XIII

APRIL, 2015

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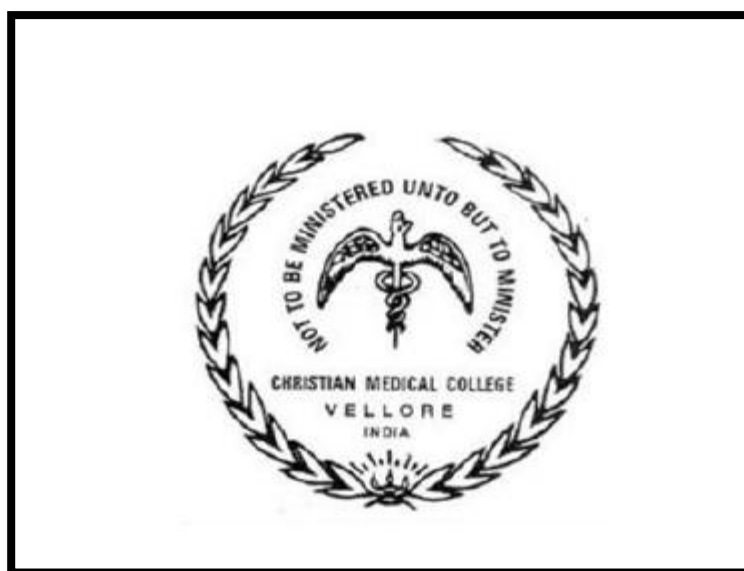
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DEPARTMENT OF BIOCHEMISTRY

CHRISTIAN MEDICAL COLLEGE

VELLORE- 632002, INDIA

DECLARATION

I hereby declare that the investigations, which form the subject matter of this study, were conducted by me under the supervision of Dr. Molly Jacob, Professor and Head of the Department of Biochemistry, Christian Medical College, Vellore.

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TABLE OF CONTENTS

INTRODUCTION	1
ABSTRACT	4
REVIEW OF LITERATURE	7
THE STUDY	51
MATERIALS	53
METHODS	55
RESULTS	72
DISCUSSION	90
CONCLUSION	98
LIMITATIONS OF THE STUDY	99
FUTURE DIRECTIONS	101
REFERENCES	102
APPENDIX	119

INTRODUCTION

Iron is an essential micro-nutrient. Cellular iron is mainly utilized for synthesis of heme, iron-sulphur clusters and ferritin. Ferritin is the storage form of iron in human beings. Iron-sulphur clusters synthesized from cellular iron are found in proteins involved in electron transport chain (Longo et al. 2011). Iron requirements vary among individuals during development (Longo et al. 2011). Excess iron produces free radicals and cause tissue damage. On the other hand, iron-deficient cells were not able to meet the energy requirements due to reduction in electron transport chain proteins. In iron deficient erythroid cells hemoglobin synthesis is also impaired (Longo et al. 2011).

Pregnancy is a normal physiological condition with increased demands for iron. Anemia is more common during pregnancy in most of the developing countries. The National Family Health Survey-3 (NFHS-3), has estimated the prevalence of anemia in pregnancy to be about 57.9 % in India (Bhat et al, 2007)

Hepcidin is the principal regulator of iron homeostasis in the body (Murray et al. 2012). It is mainly synthesized in hepatocytes. It decreases iron efflux from the cell by internalization and degradation of the iron efflux protein, ferroportin (Murray et al.

2012). Hepcidin maintains systemic iron levels and provides adequate iron to the cells when demand for iron increases.

As pregnancy progresses, there is an increased iron demand to provide adequate iron for development of the fetus. Hepcidin, which regulates systemic iron levels, has been found to be reduced during the second and third trimesters of pregnancy; this ensures adequate iron for fetal development (Finkenstedt et al. 2012). Animal studies have shown decreases in hepcidin levels through pregnancy and increases in the postpartum period (Millard et al. 2004). There is little published data in this regard in humans.

Evidence from previous studies has shown that levels of hepcidin and ferritin significantly decreased during pregnancy and also negative correlation has been shown between hepcidin and erythropoietin, a marker for increased iron demand (Finkenstedt et al. 2012). This shows the depletion of iron stores was mainly to provide more iron for fetal development (Finkenstedt et al. 2012). In the present study, we estimated serum levels of hepcidin in women with uncomplicated pregnancies and correlated this with hemoglobin and ferritin levels in these subjects. This study was done as an attempt to understand better the changes that occur in iron-related proteins in pregnancy.

KEY WORDS

Iron

Pregnancy

Hepcidin

Anemia

ABSTRACT

Serum hepcidin levels in normal pregnancy

Background of the study

Systemic iron levels are regulated by hepcidin. Evidence from animal studies shows that hepcidin levels were reduced during pregnancy, to provide adequate iron for fetal development, and raised in the post-partum period. Literature in this area on human subjects is very limited, with no data available from India

Aim of the study

The aim of the study was to determine serum hepcidin levels in women with uncomplicated pregnancies.

Materials and methods

Thirty healthy pregnant women (10 in each trimester), without any pregnancy-associated complications, were chosen as subjects. They were recruited from the antenatal clinic of the Community Health and Development (CHAD) hospital, at Christian Medical College (CMC), Vellore. Ten healthy age-matched non-pregnant females were selected as control subjects. The study protocol was approved by the Institutional Review Board of CMC, Vellore. Informed consent was obtained to collect 10ml of blood from each subject at the time

of recruitment into the study. Levels of serum hepcidin, ferritin, C-reactive protein and hematological parameters were estimated in each sample. Data were analysed by appropriate statistical tests, using SPSS, version 16. A p value of <0.05 was considered to be statistically significant.

Results

Serum hepcidin and hemoglobin levels tended to decrease as pregnancy progressed, but the changes were not statistically significant in the case of hepcidin. In the case of hemoglobin, levels in the third trimester were significantly lower than those in control subjects. Hemoglobin, serum ferritin and haematocrit levels showed significant positive correlations with one another. Hepcidin levels were not significantly correlated with any of the parameters measured.

Conclusion

Serum hepcidin levels tended to decrease with increasing gestational age, but the decreases seen were not statistically significant. Since the sample size in this study was small, further studies in this area are warranted to confirm these findings and to further study the complex biological relationships between hepcidin and iron-related proteins, in pregnancy.

REVIEW OF LITERATURE

Iron mainly occurs in two ionic forms, Fe^{2+} (bio available form) and Fe^{3+} (storage form). Iron-containing proteins are involved in various functions, such as transport of oxygen, intermediary metabolism and regulation of transcription (Longo et al. 2011). Hemoproteins in the human body that contain iron include hemoglobin, myoglobin and cytochromes (Murray et al. 2012).

The total body iron levels are about 3-4g in a normal adult male and 2-3g in a normal adult female (Ross et al,2014). In adult females, the total body iron content is less than that in males. Iron requirements increase in premenopausal women due to menstrual losses (Murray et al. 2012). Iron loss is about 1mg/day. Iron levels are maintained by absorption of about the same amount per day (Murray et al. 2012).

IRON DISTRIBUTION IN THE BODY

Hemoproteins	Adult male	Adult female
	Iron concentration	Iron concentration
	in mg	in mg
Hemoglobin	2500	1700
Myoglobin/Enzymes	500	300
Transferrin	3	3
Ferritin	600-1000	0-300

Source: Harrison's Principles of Internal Medicine, 18th Edition.

McGraw Hill; 2011; chapter 103; Page 1552

DIETARY IRON

Dietary iron mainly occurs in two forms, heme iron and non-heme iron. Heme iron is obtained from food such as red meat, fish, and poultry. Non- heme iron is obtained from plant sources (Ross et al, 2014).The bioavailability of heme iron is greater than that of non-heme iron (Ross et al, 2014). The absorption of iron is influenced by iron stores in the body. When body iron stores are low, the absorption of iron is increased; when iron stores are high, the

absorption of iron is less (Theil EC, 2004). Vitamin C reduces ferric iron in the diet to the ferrous form of iron, which is the bio-available form. Vitamin C therefore increases iron absorption (Siegenberg et al. 1991). Phytates inhibit the absorption of non-heme iron (Siegenberg et al. 1991). Calcium inhibits absorption of both heme and non-heme iron (Hallberg et al. 1991).

IRON IN ENTEROCYTES

Non-heme iron absorption from the diet

Dietary non-heme iron is absorbed in the proximal part of the small intestine (Longo et al. 2011). It is regulated at the level of absorption, as the body is not able to eliminate any excess of iron that is absorbed (Ross et al. 2014). Most of the dietary non-heme iron is in the form of ferric iron (Fe^{3+}) (Murray et al. 2012). At physiological pH and in the presence of oxygen, iron exists most commonly as a highly insoluble ferric (Fe^{3+}) form. The iron transport system in the body is able to take up unstable ferrous (Fe^{2+}) ion (Gunshin et al. 1997). In the enterocytes, the first step for absorption of iron involves reduction of insoluble ferric iron (Fe^{3+}) to soluble ferrous iron (Fe^{2+}). This is effected by a brush-border ferri-reductase (Raja et al 1992; Riedel et al. 1995; McKie et al. 2001). In mammals, the ferri-reductase activity is observed in three main

groups of proteins, they are duodenal cytochrome *b* (Dcytb/cybrd1), cytochrome *b*561 homologues and the STEAP (six transmembrane epithelial antigen of prostate) family of metallo reductases (Vargas et al. 2003)

Duodenal cytochrome b (Dcytb)

Dcytb (Cybrd1) is a plasma membrane protein with ferri-reductase activity. It is expressed in the duodenal brush border and RBC membranes. It is a member of the cytochrome *b*561 family (McKie et al. 2001). It is an iron-regulated protein that has 286 amino acids and six transmembrane domains (McKie et al. 2001). In response to iron deficiency and hypoxia, the iron- regulated Dcytb mRNA and protein are rapidly induced (McKie et al. 2001).

Ferrous iron, once formed by the action of Dcytb, enters enterocytes through divalent metal transporter 1 (DMT1) (Gunshin et al. 1997; Fleming et al. 1997), which is located on the apical surface of enterocytes.

Divalent metal transporter 1 (DMT1)

DMT1 is a member of the 'natural-resistance-associated macrophage protein' (Nramp) family. DMT1 is also called natural resistance-associated macrophage protein 2 (NRAMP2) or divalent cation transporter 1 (DCT1) or SLC11A2 (solute carrier family 11 member 2) (Longo et al. 2011). It is a 561-amino-acid protein, with 12 membrane-spanning domains. It is expressed ubiquitously. In the proximal duodenum, it mediates active transport of divalent metals such as Fe^{2+} , Cu^{2+} , Mg^{2+} (Gunshin et al. 1997). The active transport of metals is proton-coupled and mainly depends on the cell membrane potential (Gunshin et al. 1997).

Iron absorbed from the proximal duodenum (both heme and non-heme iron) forms a labile iron pool in enterocytes. When the body has sufficient iron, this pool is utilized for biosynthesis of ferritin and iron-sulphur clusters.

Iron in the ferrous form (Fe^{2+}) is exported from enterocytes by ferroportin (Fpn), which is located on the baso-lateral surface of enterocytes (McKie et al. 2000; Abboud et al 2000; Donovan et al. 2000).

Ferroportin (Fpn)

Ferroportin is a member of the metal transport protein 1 family of protein (McKie et al. 2000; Abboud et al. 2000). It is also called SLC40A1 (solute carrier family 40 member 1) or IREG1 (iron-regulated transporter 1) or MTP1 (metal transport protein 1) (Abboud et al. 2000). It is made up of 581 amino acids, with 10-12 transmembrane domains (Donovan et al. 2005). It is highly expressed in macrophages, duodenal mucosal cells, hepatocytes, Kupffer cells and placental syncytiotrophoblast cells (Abboud et al. 2000). The ferroportin gene is highly regulated. This regulation is mediated by iron-regulatory proteins (IRPs). These proteins binds to iron-responsive elements (IRE) on the 5' untranslated region (UTR) of ferroportin mRNA (Sanchez et al. 2006) and cause translational repression of ferroportin in iron deficient cell. Transcriptional activation of ferroportin mediated by the activation of heme oxygenase-1 (HO-1), occurs in macrophages following erythrophagocytosis (Delaby et al. 2008). In the duodenum, ferroportin is involved in the efflux of iron from enterocytes to blood (Murray et al. 2012).

Ferroportin is also the receptor for hepcidin, the major regulatory hormone in iron metabolism (Nemeth, et al. 2004). Hepcidin binds

to ferroportin and cause its endocytosis and degradation (Nemeth, et al. 2004) when systemic iron levels are adequate.

Hephaestin

The ferrous iron that is exported by ferroportin is converted back to ferric iron by hephaestin. This is a protein bound to the baso-lateral membrane of enterocytes (Domenico et al. 2007; Murray et al. 2012; Vulpe et al. 1999; Chen et al. 2004). It has ferroxidase activity (Vulpe et al. 1999; Roeser et al. 1970) that converts ferrous iron to ferric iron. Copper is required for structural and enzymatic activity of hephaestin (Syed et al. 2002; Vulpe et al. 1999; Chen et al. 2004). The ferric iron released from the enterocytes binds to transferrin, which transports it to target cells in the body (Schade et al. 1946).

Heme iron absorption from the diet

Heme iron is absorbed in the proximal part of the small intestine. It is thought to be absorbed via a heme transporter, heme carrier protein 1 (HCP 1) (Latunde-Dada et al. 2006).

Heme carrier protein 1 (HCP 1)

HCP 1 belongs to the family of proton-coupled transporter/major facilitator super- family (Blanc L et al, 2012). HCP 1 is also called as proton coupled folate transporter (PCFT) (Blanc L et al, 2012). HCP-1/PCFT is mainly a folate transporter and low-affinity heme transporter that is regulated by cellular iron levels (Laftah et al. 2009). HCP-1 undergoes post-translational regulation, by iron levels in the duodenum (Latunde-Dada et al. 2006). Iron deficiency with reduced iron levels causes localisation of HCP-1 on the apical surface of enterocytes. Excess of iron results in localization of HCP-1 in the cytoplasm (Blanc L et al, 2012)

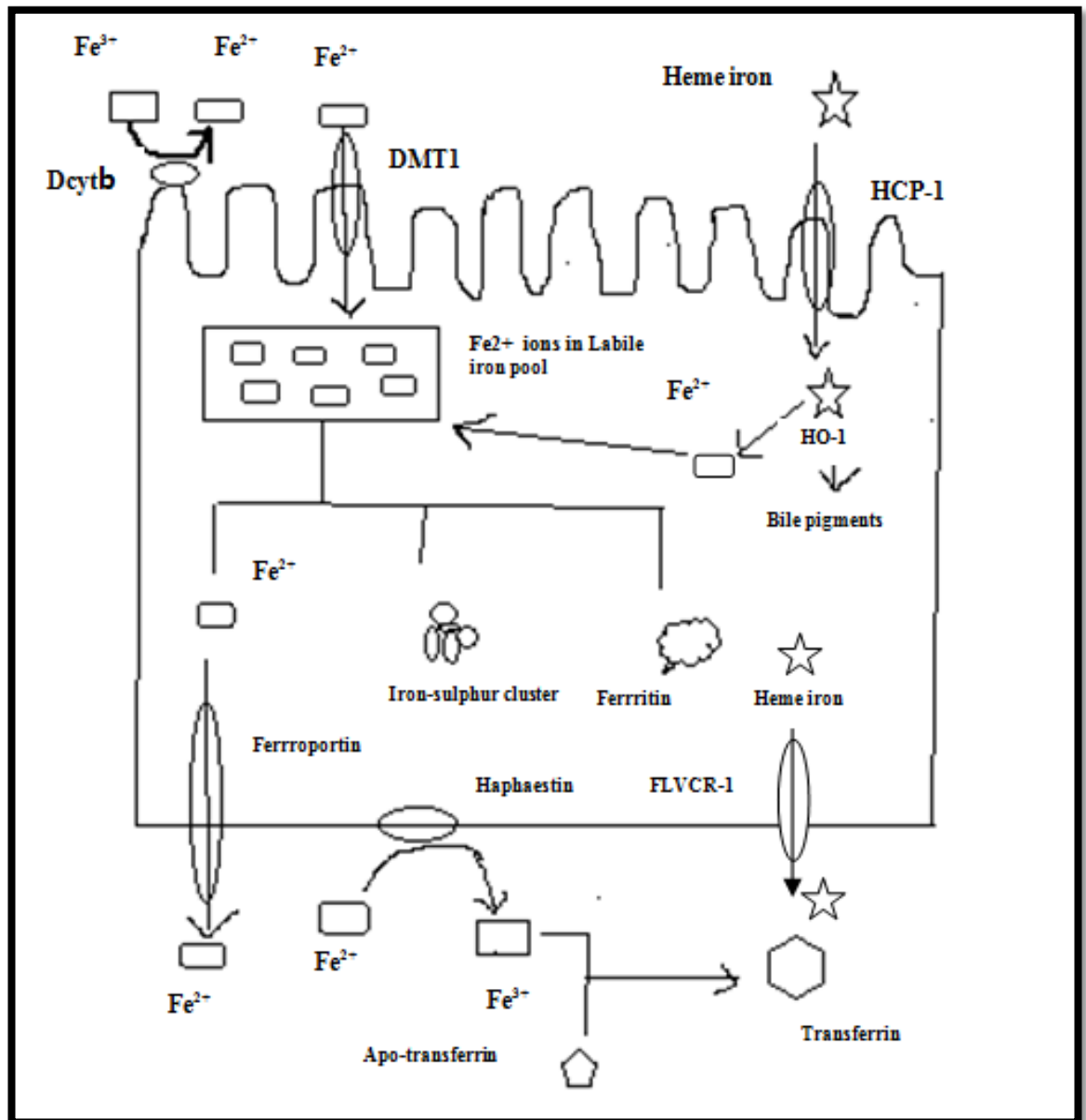
Absorbed heme iron is acted upon by hemoxygenase-1 (HO-1) in the enterocytes to release iron (Raffin et al. 1974). Fe^{2+} iron released by this enzyme adds it to the labile iron pool. Labile iron pool serves as a source for synthesis of ferritin (storage form), iron-sulphur cluster and heme proteins. Heme iron is exported out of enterocytes

via feline leukemia virus subgroup C cellular receptor 1 (FLVCR 1) (Quigley et al. 2004).

Feline leukemia virus subgroup C cellular receptor 1 (FLVCR 1)

FLVCR exports cytoplasmic heme. It is important for erythroid differentiation (Keel et al. 2008). FLVCR is expressed more at sites where high heme flux is needed. FLVCR 1 is also expressed in various tissues such as the liver, erythropoietic progenitor cells, duodenum, kidney, placenta and brain (Keel et al. 2008).

Figure1- Absorption of heme and non heme iron in duodenal enterocytes



IRON IN CIRCULATION

Ferric iron released from enterocytes is taken up by transferrin (Tf), (Schade et al. 1946), the major iron transporter in circulation.

Transferrin

Transferrin is a glycoprotein synthesized in the liver (Longo et al. 2011). Human transferrin contains 678 amino acid residues and two asparagine-linked glycans. It contains two iron-binding sites for ferric iron (Aisen et al. 1966). Normally, one-third of iron binding sites in transferrin are bound to iron, resulting in transferrin saturation levels of 30-50% (Longo et al. 2011) . The half-life of transferrin-bound iron is about 60-90 minutes. This iron taken up by cells are utilized or stored as ferritin (Ross et al. 2014). Transferrin with ferric (Fe^{3+}) iron bound to it is called holo-transferrin (Tf) (Murray et al. 2012). Cells take up holo-transferrin via the transferrin receptor (TfR) (Murray et al. 2012).

Non-transferrin-bound iron (NTBI)

When serum iron levels exceed the iron-binding capacity of transferrin, iron in circulation forms non-transferrin-bound iron (NTBI) (Barisani et al. 1995).

Transferrin receptors

Transferrin receptors are a cell surface glycoprotein present in all cells, except mature red blood cells (Ross et al. 2014). TfR is highly expressed in erythroid marrow , placenta (Wada et al. 1979) and the liver (Gatter et al. 1983). The transferrin receptor is a disulfide-linked homodimer. Each monomer has 760 amino acids and has a short cytoplasmic N-terminal region, a single transmembrane domain and a glycosylated extracellular region. The ligand-binding domain is extracellular. The intracellular segment has a phosphorylation site activated by protein kinase C (Davis et al. 1986).

Transferrin receptors are of two types, TfR1 and TfR2 (Kawabata et al. 1999). TfR1 is mainly involved in cellular uptake of transferrin bound iron. TfR1 has high affinity for transferrin bound iron when compared to TfR 2 α (Kawabata et al. 2001; Ikuta et al. 2010). TfR2 is highly expressed on hepatic cells and erythroblasts (Kawabata et al. 2001). It has two isoforms TfR2 α and TfR2 β (Kawabata et al. 1999).TfR2 α is a transmembrane protein whereas TfR2 β is an intracellular protein (Kawabata et al. 1999).

Cellular uptake of iron (transferrin cycle)

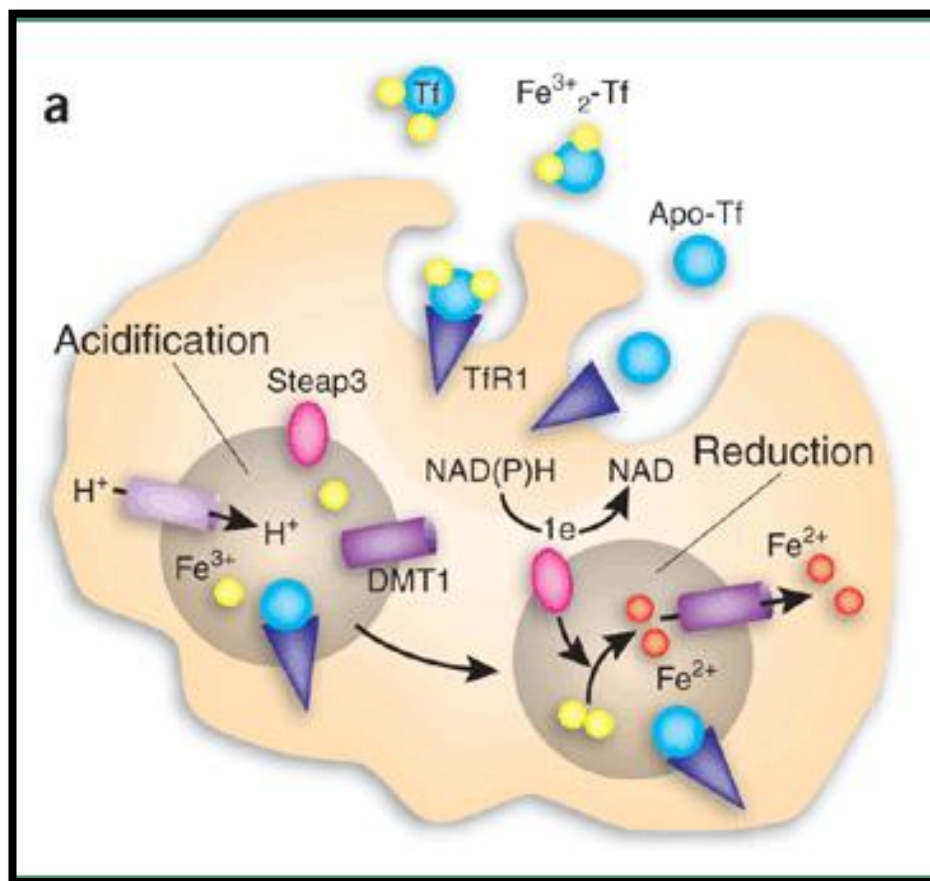
The transferrin cycle occurs during cellular uptake of transferrin-bound iron (Murray et al. 2012). Cellular uptake occurs by the process of receptor-mediated endocytosis (Ross et al. 2014). When holo-transferrin binds to TfR, the Tf-TfR complex is endocytosed. The interior of the endosome formed is acidic. Due to the low pH, the ferric iron is released from the Tf-TfR complex. This iron is then reduced to its ferrous form by STEAP3 (six transmembrane epithelial antigen of prostate family member-3) (McKie et al. 2005). STEAP3 is a NADPH-dependant ferri-reductase (Ohgami et al. 2005). The ferrous iron formed exits the endosome through DMT1 in the endosomal membrane and enters the cell cytosol. Transferrin and transferrin receptor are recycled back to the cell surface for further uptake of iron. The transferrin cycle occurs in a cell about 10-20 times a day (Murray et al. 2012).

Soluble transferrin receptor (sTfR)

Soluble transferrin receptor (sTfR) is a proteolytic product of TfR1, which circulates in plasma (Ross et al. 2014). Proteolysis occurs at surface of the receptor forming sTfR, which is released by exocytosis into the blood (Beguin 2003). About 80% iron in circulation is

utilized by erythroid precursor cells (Longo et al. 2011). The circulating sTfR is directly proportional to expression of cellular TfR (Beguin 2003). The quantity of transferrin receptor expressed on a cell surface depends on its need for iron. sTfR is increased in iron-deficiency anemia (Longo et al. 2011). sTfR is a good marker of maternal iron status and fetal demand and is increased during the course of normal pregnancy (Choi et al. 2000).

Figure 2: Transferrin cycle



Source: Mckie et al, 2005

Uptake of non-transferrin-bound iron (NTBI)

Uptake of non-transferrin-bound iron by cells causes iron toxicity (Batey et al. 1981). The process of NTBI uptake in liver occurs mainly via DMT1 and Zrt- and Irt-like protein, 14 (ZIP 14), a zinc transporter (Arias et al. 2011). The transporters involved in such uptake in cardiac myocytes have been shown to be L-type of Ca^{2+} channels (Oudit et al. 2003) and transient receptor potential cation channel (TRPC 6) (Mwanjewe et al. 2000). The toxicity due to uptake of NTBI occurs in hepatocytes, cardiac myocytes and endocrine tissues (Andrews NC et al. 2007).

Regulation of cellular iron homeostasis

Increased free iron in the cell leads to oxidative damage (Murray et al. 2012). Iron regulatory proteins, IRP1 and IRP2, are cytosolic RNA-binding proteins that help maintain iron level inside the cell. IRP1 and IRP2 regulate post transcriptional expression of mRNA of proteins involved in iron utilization (Theil EC 1993). Iron responsive elements (IRE) are located in the 5' untranslated region (UTR) in the mRNA of ferritin (Leibold et al. 1988), mitochondrial aconitase (Zheng et al. 1992) and erythroid δ -aminolevulinic acid

synthase (δ -ALAS) (Dandekar et al. 1991). IRE are also located at 3' end of mRNA of TfR1 and DMT1 (Dupic et al. 2002).

In iron deficiency, IRP is involved in translational control by binding to IRE at the 5'UTR of ferritin and ferroportin, to reduce synthesis by translational repression (Ross et al. 2014). Binding of IRP to 3' UTR of TfR1 and DMT1 increase the mRNA stability to enhance iron transport (Ross et al. 2014).

Iron regulatory protein 1 (IRP 1)

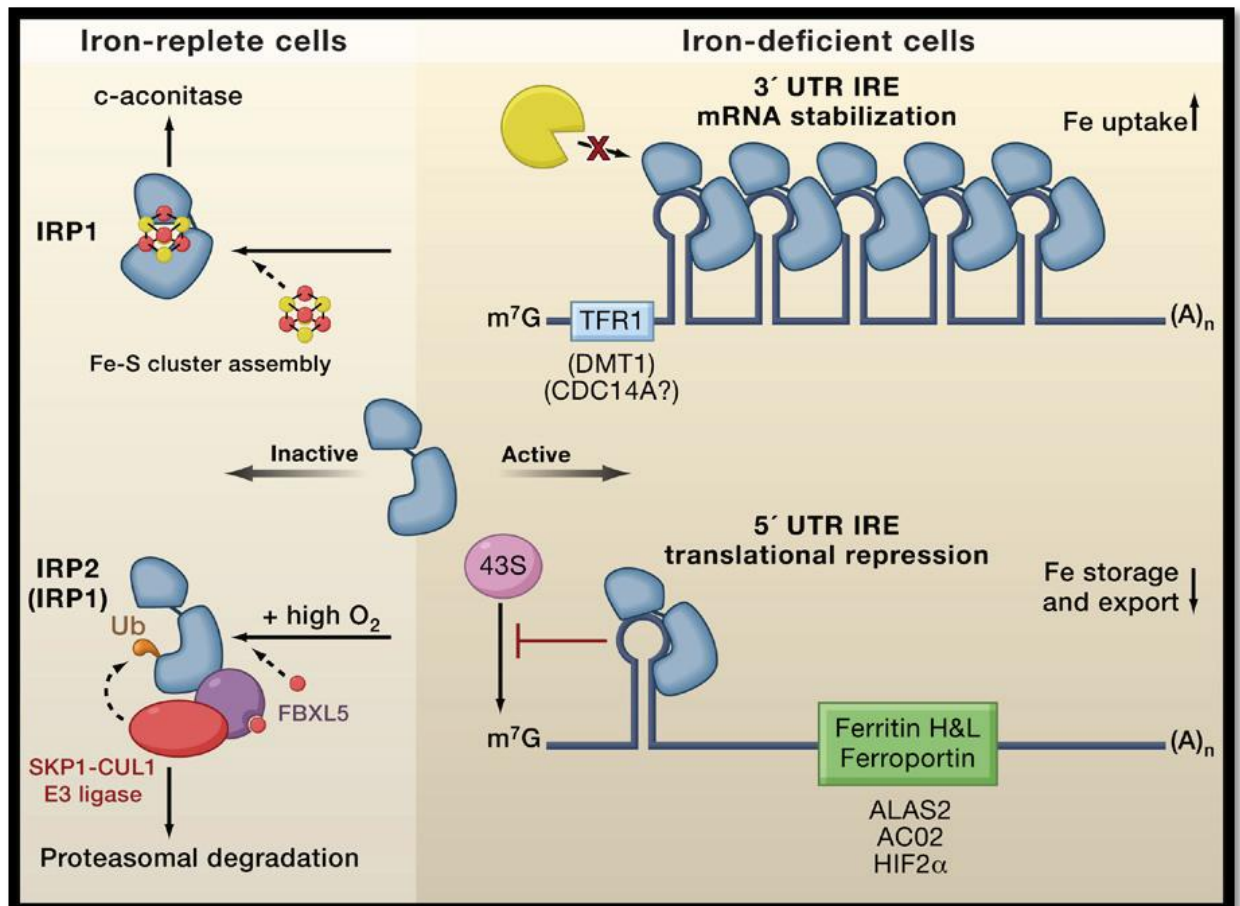
IRP 1, an iron sulphur cluster protein (Rouault et al. 1991) has two functions. In iron- replete cells, it functions as cytoplasmic aconitase (that converts citrate to isocitrate) (Rouault et al. 1992). Both IRP 1 and 2 have equal affinity (Rouault et al. 1991) for IRE. In iron-deficient cells, binding of IRP1 to IRE-containing TfR1 mRNA at the 3' UTR stabilizes TfR1 mRNA (Müllner et al, 1989; Casey et al. 1989). Binding of IRP to the 5' UTR of ferroportin and ferritin, cause translational repression (Goossen et al. 1990) .

Iron regulatory protein 2 (IRP 2)

IRP2 is an iron-sulphur cluster protein (Rouault et al. 1992). It functions only as RNA-binding protein. When iron is scarce, IRP2

binds to IRE; when intracellular iron levels are increased, IRP2 undergoes proteasomal degradation.

Figure 3: Regulation at cellular level



Source: Hentze et al, 2010.

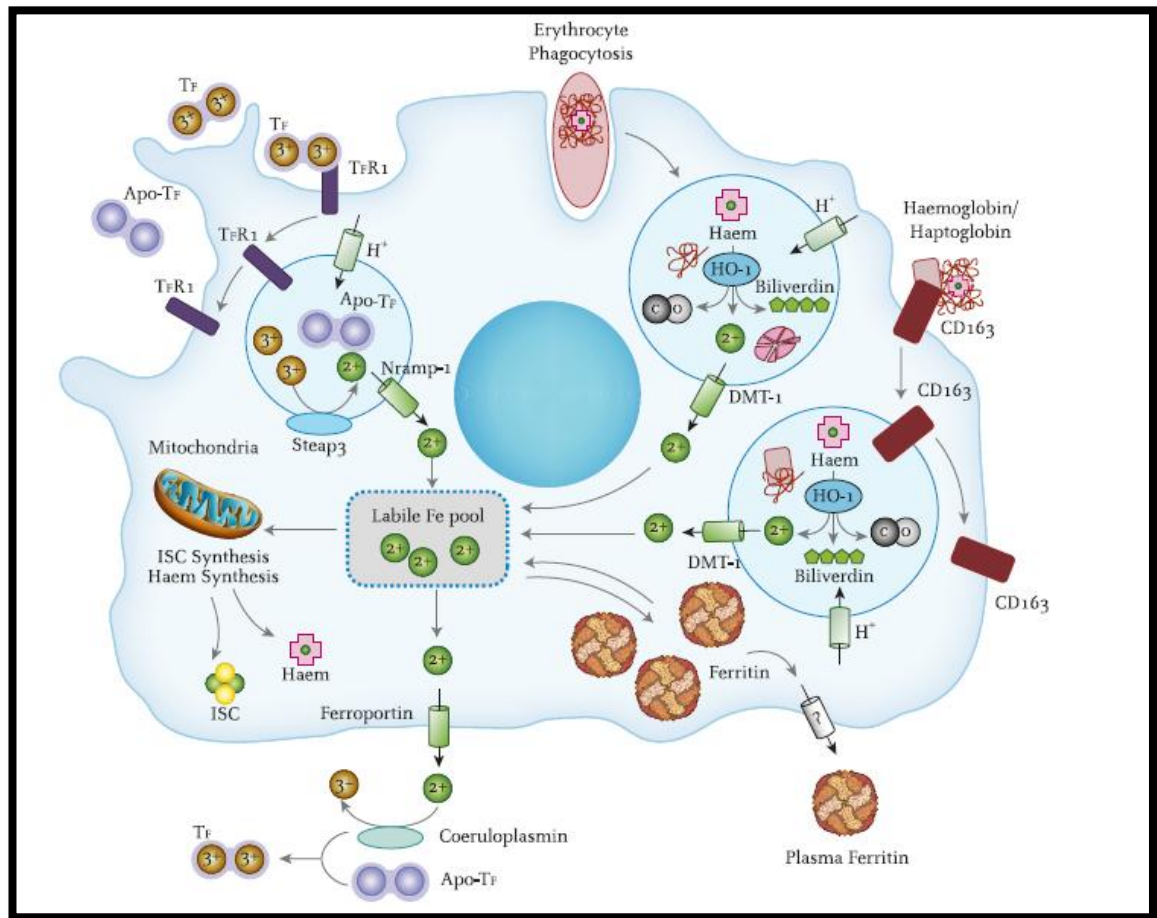
IRON IN MACROPHAGES

Most of the iron in erythrocytes is recycled from the monocytes and macrophages of the reticuloendothelial system; this is 20 times more than the amount absorbed from the intestine (Andrews NC 1999).

The reticulo-endothelial system recycles iron (Andrews NC 1999). Iron enters the macrophages by three mechanisms (Evstatiev R et al.2012)

1. Phagocytosis of senescent erythrocytes
2. Heme uptake
3. TfR1-mediated uptake of holo-transferrin

Figure 4: Iron in macrophages



Source: Evstatiev R et al, 2012.

Phagocytosis of senescent erythrocytes in macrophages

Senescent erythrocytes are taken up by phagocytosis of macrophages. As a result of phagocytic activity, heme iron is released from the erythrocytes. Extracellular free heme is toxic because of its oxidant property (Ross et al. 2014). Heme is then

acted on by heme oxygenase-1 (Raffin et al. 1974). The iron released by this enzyme adds to the labile iron pool. This pool is maintained in at physiological levels by IRP-dependent translational control of ferritin and transferrin receptor. IRP-independent ferritin degradation also occurs to prevent toxic effects (Konijn et al. 1999). Some of the heme iron is exported from macrophages via feline leukemia virus subgroup C cellular receptor 1 (FLVCR 1) (Quigley et al. 2004).

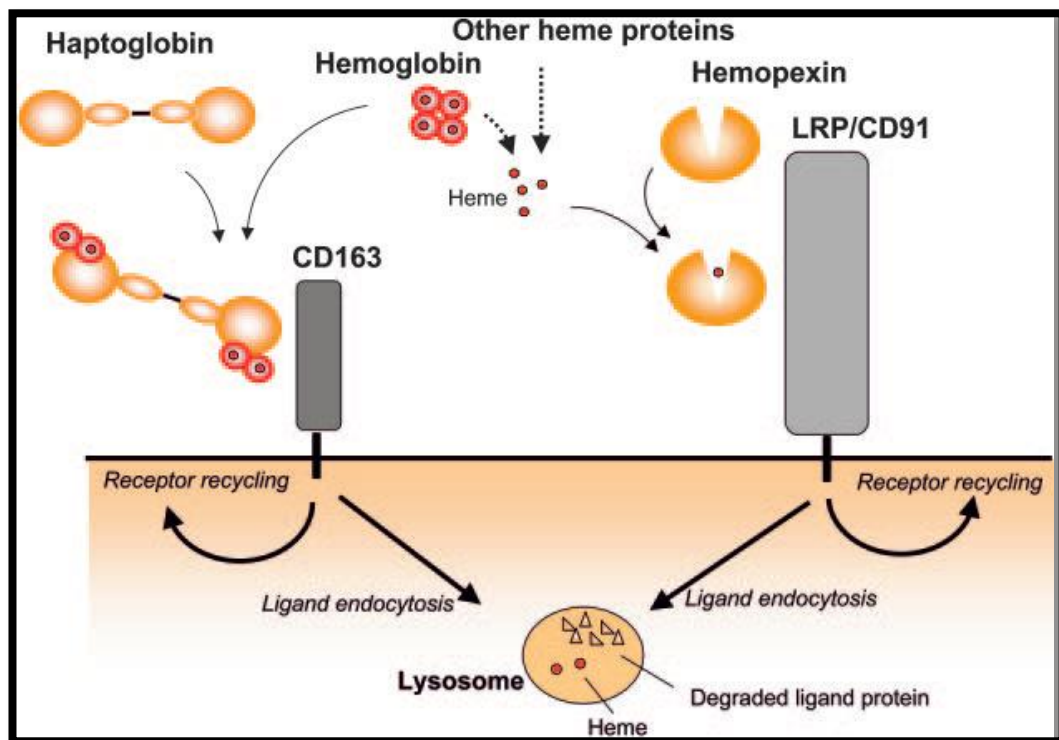
Heme uptake by macrophages

The forms in which heme is taken up by macrophages are hemoglobin and extracellular heme, which is produced as a result of haemorrhage, hemolysis or other types of cell damage. Heme in the form of hemoglobin is taken up by macrophages via a heme-scavenging receptor, CD163, as hemoglobin-haptoglobin complexes (Kristiansen et al. 2001). CD163 is located most commonly in phagocytic macrophages (Kristiansen et al. 2001).

Extracellular heme binds to hemopexin, a β 1-glycoprotein with high affinity for heme (Hrkal,et al. 1974). The heme-hemopexin complex is taken up by macrophages through LRP/ CD91 (LRP-low density lipoprotein receptor-related protein). LRP/ CD91, a α 2-macroglobin receptor, is expressed in macrophages, hepatocytes, adipocytes,

neurons and syncytiotrophoblasts (Moestrup et al. 1992). Heme-hemopexin complex and hemoglobin-haptoglobin complex, along with the receptor complex, are endocytosed. In the lysosome, the ligand gets degraded to release heme. The receptors are recycled back to the surface (Hvidberg et al. 2005).

Figure 5: Heme uptake in macrophages



Source: Hvidberg V et al, 2005

TfR1-mediated uptake of transferrin by macrophages

TfR1 mediated uptake in macrophages occurs via receptor-mediated endocytosis (Figure 4).

Ferrous iron released from heme, transferrin and senescent erythrocytes forms the labile iron pool. Iron from labile iron pool is used for ferritin synthesis or is exported via ferroportin. Ceruloplasmin reduces ferric Fe^{3+} to ferric Fe^{2+} (Roeser et al. 1970), which then binds to transferrin which transports iron to target tissues. The process by which ferritin is released from macrophage is still unclear (Barton et al. 1999)

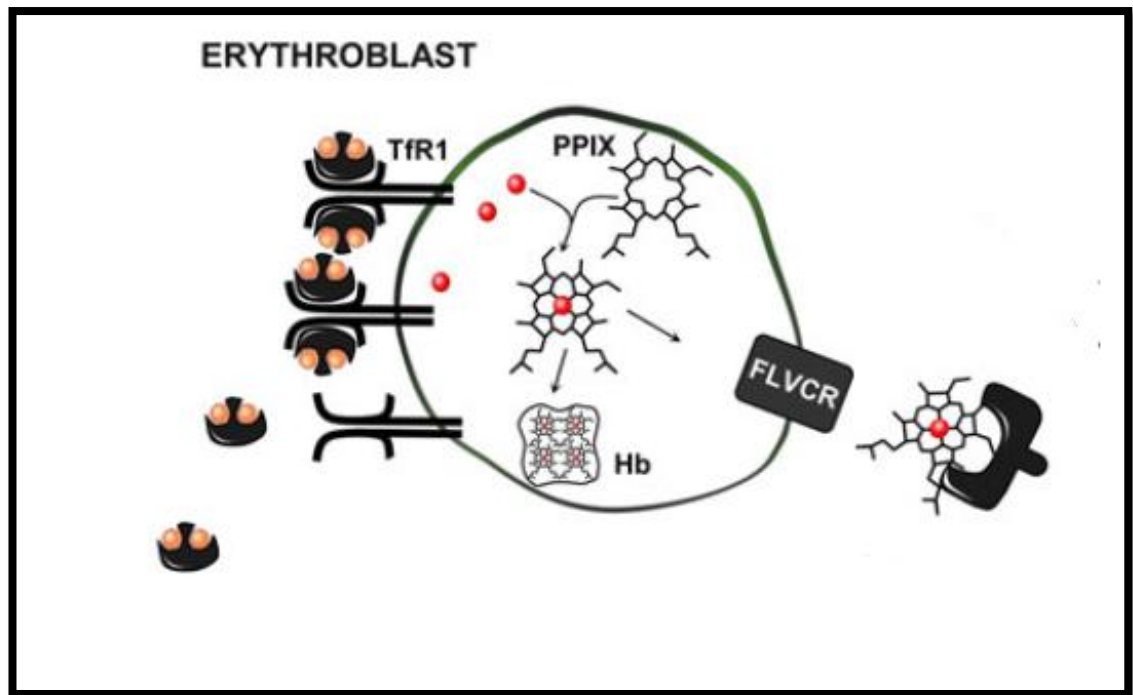
IRON IN ERYTHROBLAST

Erythroblast take up a large quantity of iron (approximately 80%) for hemoglobin synthesis (Longo et al. 2011). Iron enters erythroblast through TfR1 (Longo et al. 2011). STEAP3, an endosomal ferrireductase mainly found in erythroid cells. (Ohgami et al.2005).

Ferrous iron is utilized for hemoglobin synthesis. Excess iron can be stored in the cell as ferritin (Longo et al. 2011). FLVCR expressed in

erythropoietic progenitor cells exports out hemoprotein (Keel et al. 2008).

Figure 6: Iron in erythroblast



Source: Lipinski P et al, 2013

IRON IN HEPATOCYTES

Ferritin is a ubiquitous protein and the main storage form of iron (Murray et al. 2012). Iron is stored as ferritin mainly in hepatocytes, macrophages in the spleen and bone marrow (Worwood 1979).

Ferritin

Mammalian ferritin is a large protein. It is about 450kDa in size and made up of 24 units of apoferritin monomers to form a spherical particle. It encloses a core of ferric-hydroxy-phosphate, which can hold up to 4,000 atoms of iron as Fe^{3+} (Arosio et al. 1978). It has two subunits heavy (H-chain) and light chains (L-chain), which contain 4000 atoms of iron (Harrison et al. 1996). The proportion of H and L subunits varies in different tissues. The L form predominates in the liver and spleen and the H form predominates in the heart and kidney (Torti et al. 2002).

Ferritin is a soluble protein. When degraded, it accumulates in lysosomes as insoluble haemosiderin. Both ferritin and haemosiderin are storage forms of iron (Theil EC 1990). Circulating ferritin is mostly in the L form; it does not contain iron (Theil EC 1990). H-ferritin subunits have an active ferroxidase site in human (Theil EC 1990).

Hepcidin

Hepcidin, the major regulator of systemic iron is produced in liver as a pre-propeptide, with 84 amino acids. It contains a 24 amino acid N-terminal endoplasmic reticulum-targeting signal sequence (Hunter et al. 2002). The 60-amino acid pro-hormone is produced after cleavage of N-terminal endoplasmic reticulum-targeting signal sequence (Park et al. 2001). The pro-hormone has pro-protein convertase activity that cleaves it and produces mature hepcidin with 25, 20 and 22 amino acids (Pigeon et al. 2001; Park et al. 2001; Krause et al. 2000). The active form is hepcidin with 25 amino acids (Park et al. 2001; Krause et al. 2000). Hepcidin in circulation is bound to α 2-macroglobin (Peslova et al. 2009). It is excreted by the kidney (Park et al. 2001; Krause et al. 2000).

Hepcidin has eight cysteine residues with four disulphide cross-links and a beta hairpin structure (Park et al. 2001). It has a highly conserved N-terminal arm, essential for the hormone activity (Park et al. 2001). Hepcidin regulates iron homeostasis by binding to ferroportin. Ferroportin is then internalized and degraded (Nemeth et al. 2004). Hepcidin is the protein mainly involved in the regulation of iron at the level of intestinal absorption, recycling of iron in macrophages and also in mobilization of iron from hepatic stores (Nemeth et al. 2004).

Regulation of hepcidin

The transcription of hepcidin, which regulates systemic iron balance, is mainly controlled by the following factors:

1. Transferrin saturation
2. Liver iron
3. Erythroid activity
4. Inflammation
5. Hypoxia

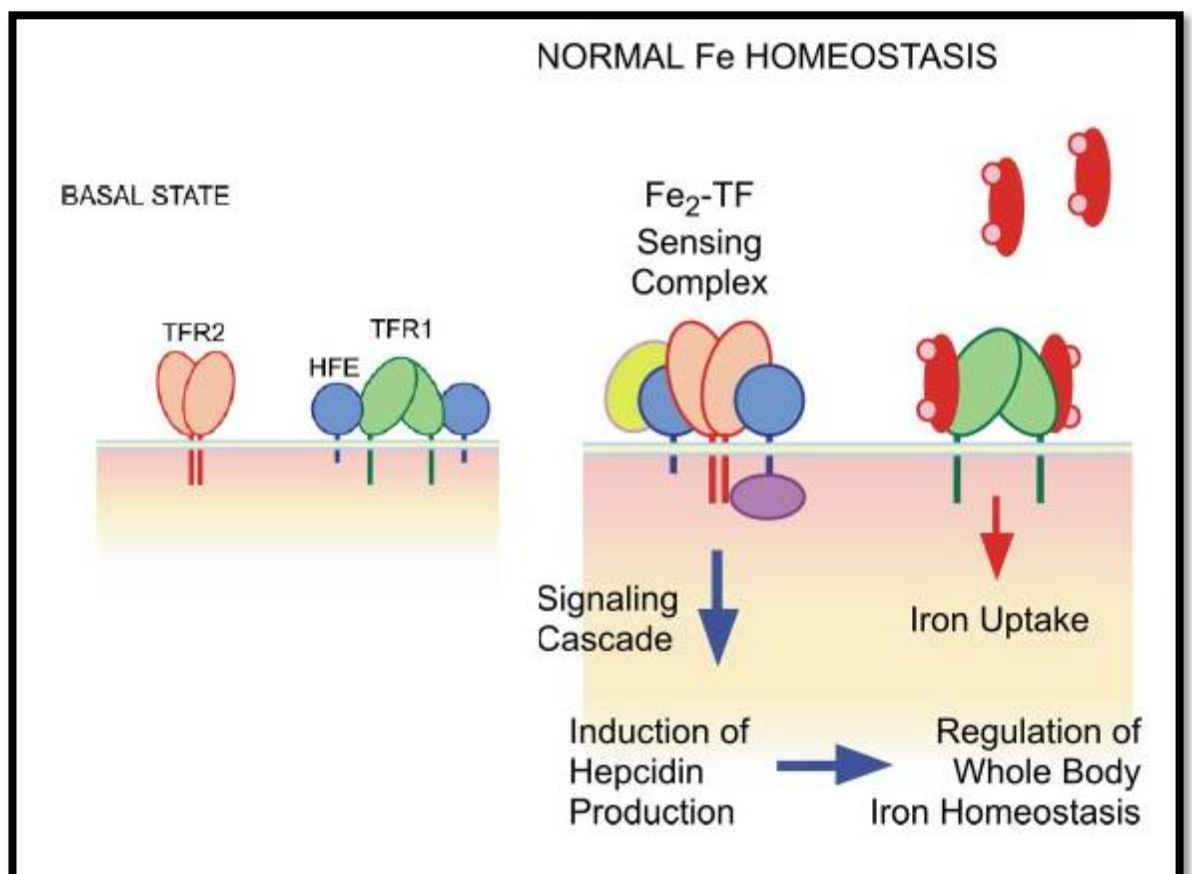
Hepcidin is up-regulated by iron over-load and inflammation and down-regulated by erythropoiesis and hypoxia (Nemeth et al. 2004; Pigeon et al. 2001; Nicolas et al. 2002).

Hfe-TfR2 pathway in regulation of hepcidin

The Hfe gene is an atypical major histocompatibility complex class-1 like molecule. The protein, when mutated, leads to classical hereditary hemochromatosis (Goswami et al. 2006). Hfe is associated with β 2-microglobulin. It is expressed on the cell surface, associated with transferrin receptor 1 (TfR 1) (Feder et al. 1998). The transferrin receptor 1 has binding sites for transferrin and Hfe. Transferrin competes with Hfe for binding sites on TfR1, as binding sites of both transferrin and Hfe overlaps (West et al. 2001).

Hfe can also bind to transferrin receptor 2 (TfR2) (Goswami et al. 2006). When the concentration of holo-transferrin in blood is increased, it displaces Hfe from TfR1 and favours Hfe-TfR2 interaction. Hfe-TfR2 binding causes activation of p38-MAPK (microtubule associated protein kinase- extracellular signal-regulated kinase) signalling cascade (Wallace et al. 2009). This promotes hepcidin transcription (Goswami et al. 2006).

Figure 7: Role of Hfe-TfR2 in hepcidin transcription



Source: Goswami et al, 2006.

BMP-SMAD signalling pathway for hepcidin regulation

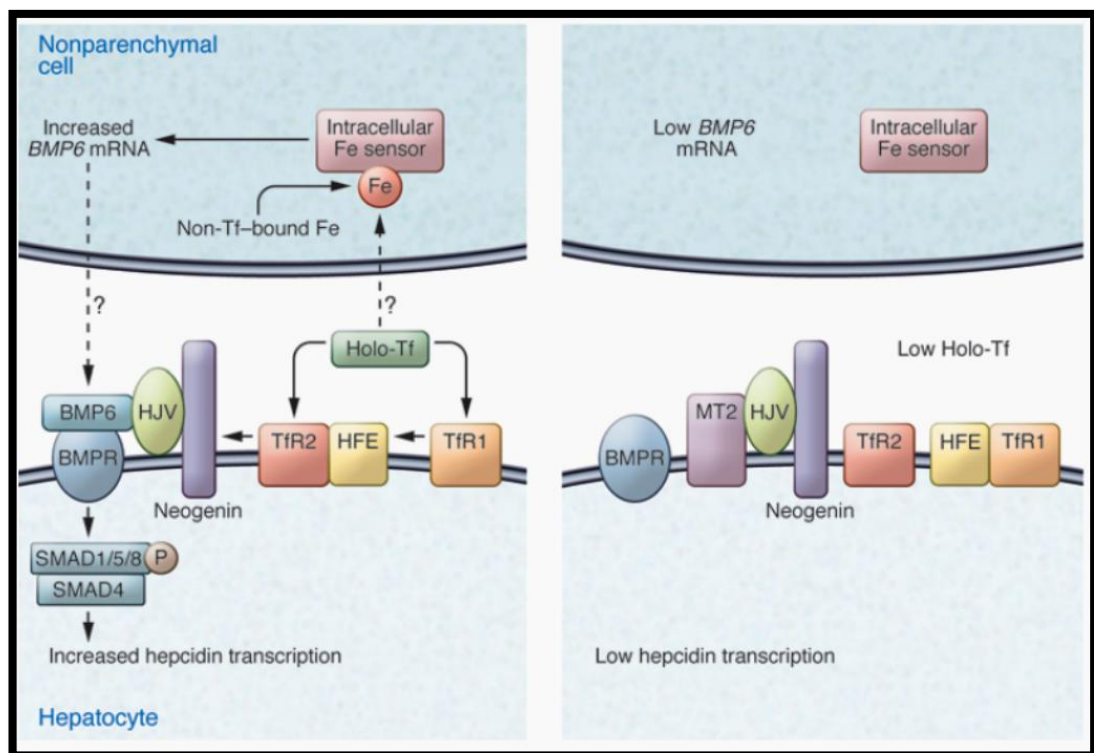
The BMP receptor complexes are serine/ threonine kinase receptors. The BMP receptor complex is composed of type I (BMPRI) and type II (BMPRII). When a ligand binds to the receptor complex, BMPRII phosphorylates BMPRI which then propagates the signal to the cytosolic transcription factors SMAD 1/5/8 (receptor-activated SMAD, R-SMAD). Increase in hepcidin expression through a number of BMPR ligands such as BMP2, BMP5, BMP6, BMP7 and BMP9 is known (Wang et al. 2005). Among these BMPs, the key regulator of hepcidin expression is BMP6 (Andriopoulos et al. 2009). BMP 6 is produced from non-parenchymal cells in the liver in response to increase intracellular iron.

The BMP binds to BMP receptors to induce phosphorylation of receptor-activated SMAD (R-SMAD). SMAD protein is a human homolog of *Drosophila melanogaster* MAD protein (Mothers-Against-Decapentaplegic) and *Caenorhabditis elegans* SMA gene. The activation of R-SMAD causes activation of transcriptional complexes by SMAD4 by dimerization with SMAD4 and translocates into the nucleus causing hepcidin induction (Wang et al. 2005).

Hemojuvelin (HJV) is a glycosphosphatidyl inositol (GPI)-anchored protein, which acts as a bone morphogenetic protein (BMP) co-

receptor. Hemojuvelin also occurs as two isoforms- membrane bound and soluble form. HJV acts through BMP-SMAD signalling for regulation of iron (Babitt et al. 2006). Both HJV and Hfe activate hepcidin transcription via R-SMAD phosphorylation and SMAD4 (Corradini et al. 2009; Kautz et al. 2009). Soluble hemojuvelin (sHJV) is a negative regulator of hepcidin mRNA transcription (Lin et al. 2005).

Figure 8: Model for hepcidin transcription (BMP-SMAD pathway)

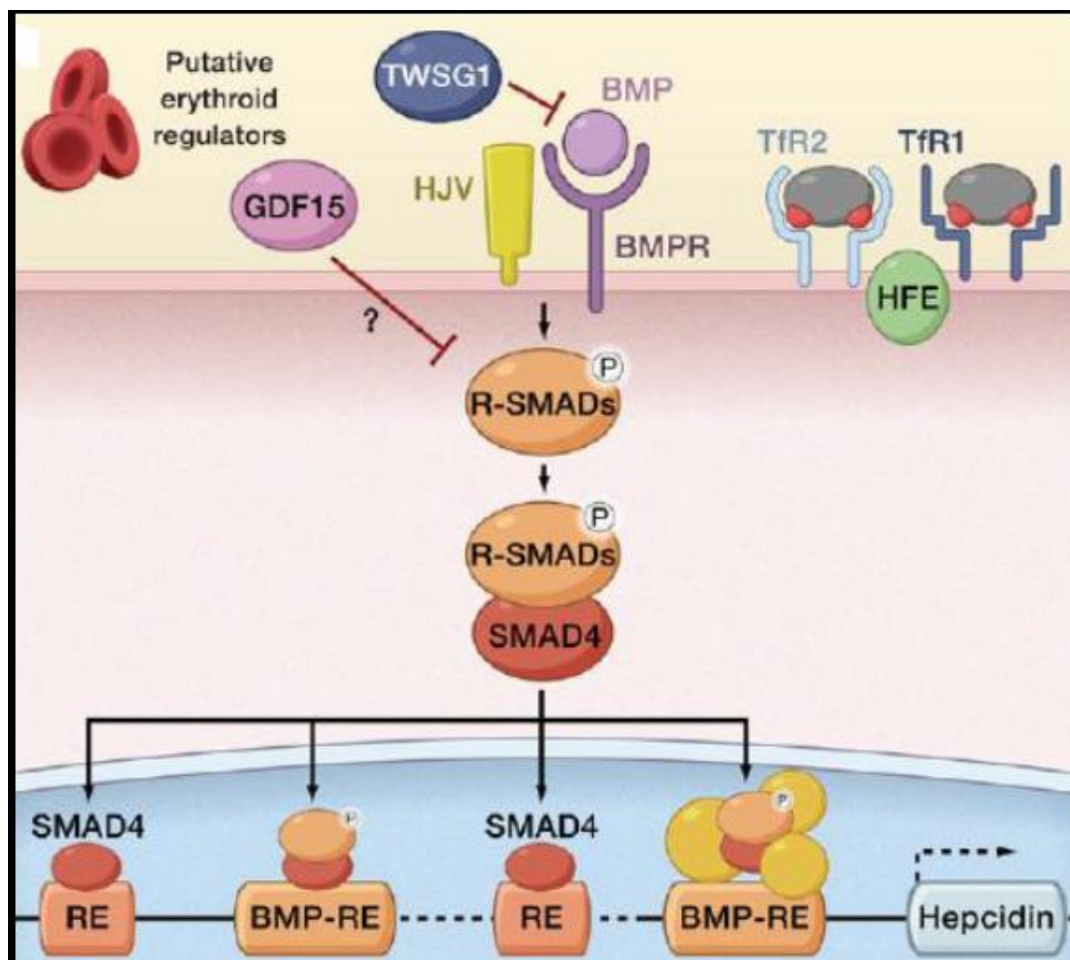


Source: Zhao et al .2013

2. GDF 15 (growth differentiation factor) and TWG 1 (twisted gastrulation factor) in regulation of hepcidin

The down-regulation of hepcidin by erythropoiesis is mediated by factors that are currently not definitively known. Erythroferrone (ERFE), GDF 15 (growth differentiation factor) and TWSG 1 (twisted gastrulation factor) are candidate molecules released by the bone marrow that could mediate this effect (Hentze et al, 2010).

Figure 9: Erythroid regulation of hepcidin

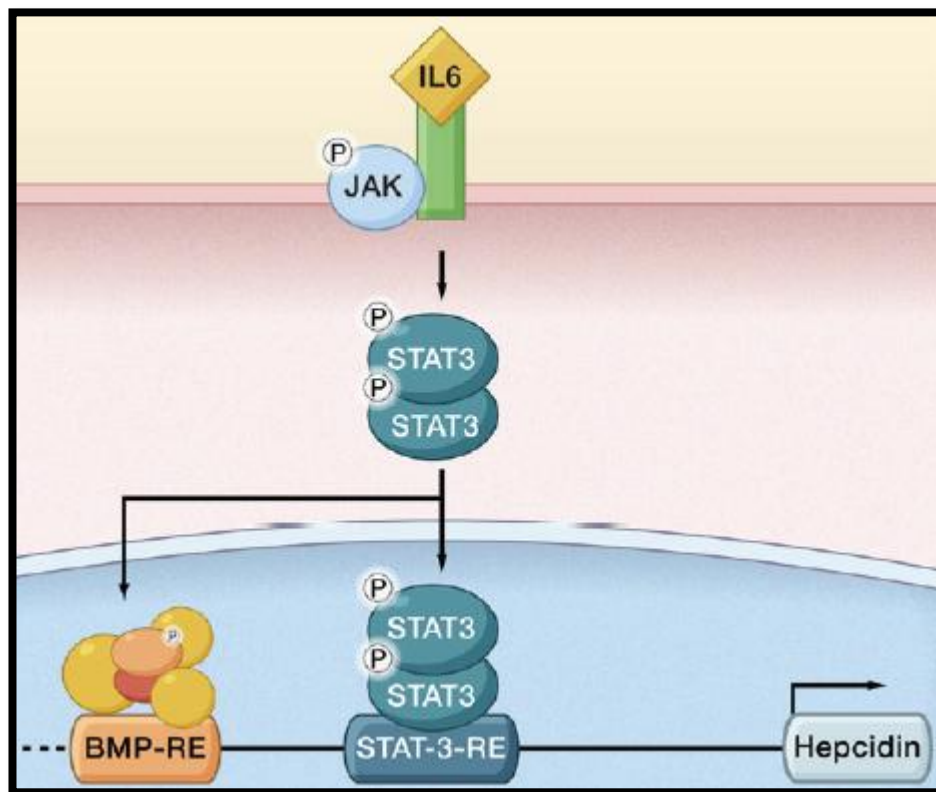


Source: Hentze et al, 2010.

3. Role of inflammatory cytokines in regulation of hepcidin

The inflammatory cytokine, interleukin-6 (IL6) activates JAK/STAT (JAK-Janus kinase; STAT- signal transducer and activator of transcription). STAT 3 activates the hepcidin promoter by binding to the STAT-binding motif close to the hepcidin transcription start site and up-regulates its expression (Nemeth et al. 2004). BMP signalling is also activated in response to inflammation via SMAD4 (Wang et al. 2005) and up-regulates hepcidin expression.

Figure 10: Regulation of hepcidin by inflammatory cytokine



Source: Hentze et al, 2010.

4. Role of hypoxia in regulation of hepcidin

Erythropoietin produced in response to hypoxia activates erythropoiesis. This, in turn, suppresses hepcidin.

Role of hepcidin in iron-related disorders

Iron-related disorders are mainly due to iron over-load and iron deficiency. It was observed that disorders with iron over- load are commonly associated with hepcidin deficiency or resistance to hepcidin action (Ganz et al, 2011). Hereditary hemochromatosis is a genetic disorder associated with iron metabolism (Longo et al. 2011). This genetic disorder is associated with mutation in Hfe gene. Juvenile form of hemochromatosis is mainly associated with non-Hfe gene mutations (mutation in hemojuvelin and hepcidin genes). In adults with hemochromatosis, hepcidin synthesis is partially responsive to iron overload. Inappropriate levels of hepcidin were observed with iron over-load. Conventional treatment for hemochromatosis, venisection reduces the iron levels in circulation and cause hepcidin deficiency (Spasic V et al. 2007).

In β -thalassemia, the expansion of erythroid precursors is observed mainly in the bone marrow. The defect in β -globin production in erythroid precursors leads to excess α -chains. This excess α -chain

will precipitate that leads to the apoptosis of erythroid precursors in the marrow. The increase in erythropoietin production due to anemia leads to massive ineffective erythropoiesis which causes suppression of hepcidin leading to increased iron absorption(Tanno et al. 2007; Tanno et al. 2009).

A genetic disorder, iron-refractory iron deficiency anemia (IRIDA), due to mutations in a negative regulator of hepcidin, the membrane protease matriptase-2 (also called TMPRSS6 - transmembrane protease serine 6) which cleaves HJV, reducing the phosphorylation of the SMAD complex (Finberg et al. 2008; Du et al. 2008). This disorder is associated with over expression of hepcidin despite lower levels as in iron deficiency anemia

Chronic inflammatory conditions such as infection, rheumatological disorders and inflammatory bowel disease are associated with iron deficiency anemia. In these conditions, anemia is partly due to increase in hepcidin levels by IL-6 stimulation (Ross et al. 2014).

In chronic kidney disease, high levels of serum hepcidin levels were seen. Increased serum hepcidin levels were due to decrease in clearance or due to inflammation associated with the underlying disease and hemodialysis (Zaritsky et al. 2009). High levels of hepcidin lead to reduction in iron supply to erythroid precursors

leading to anemia and decreased responsiveness to erythropoietin therapy (Zaritsky et al. 2009).

Anemia is a common manifestation of malignancy. Studies on anemia associated with Hodgkin's disease and multiple myeloma has shown that features were similar to anemia of inflammation with high levels of hepcidin (Hohaus et al. 2010; Sharma et al. 2008). Increased hepcidin levels mainly due to inflammatory cytokines.

Variation in hepcidin levels leads to change in the systemic iron levels. Increased hepcidin is associated with low serum iron levels, the iron supply to the erythroid bone marrow decrease leading to decreased heme synthesis. This reduction in heme synthesis manifests as anemia.

Therapeutic implications of hepcidin

Hepcidin levels are reduced in hereditary hemochromatosis and iron loading anemia with inefficient erythropoiesis (Spasic V et al. 2007). Conventional treatment for hereditary hemochromatosis, phlebotomy is ineffective since it cause inappropriate decrease in hepcidin levels.

Therapeutic intervention with hepcidin antagonists can be considered in hereditary iron-refractory iron deficiency anemia. In this condition increased levels hepcidin is due to mutation in the membrane protease matriptase-2 (also called TMPRSS6) (Finberg et al. 2008; Du et al. 2008), treatment with hepcidin antagonist can prevent resistance developed against oral iron supplementation .

Anemia in chronic kidney disease is associated with elevated hepcidin levels. Treatment with hepcidin antagonist can be considered in the treatment for anemia in chronic kidney disease.

PREGNANCY

Pregnancy is a normal physiological process, which extends over a period of 280 days or 40 weeks from a woman's last menstrual period to child birth. The period of 40 weeks is divided into first, second and third trimesters. Each trimester comprises a period of 13 weeks (Cunningham et al. 2014).

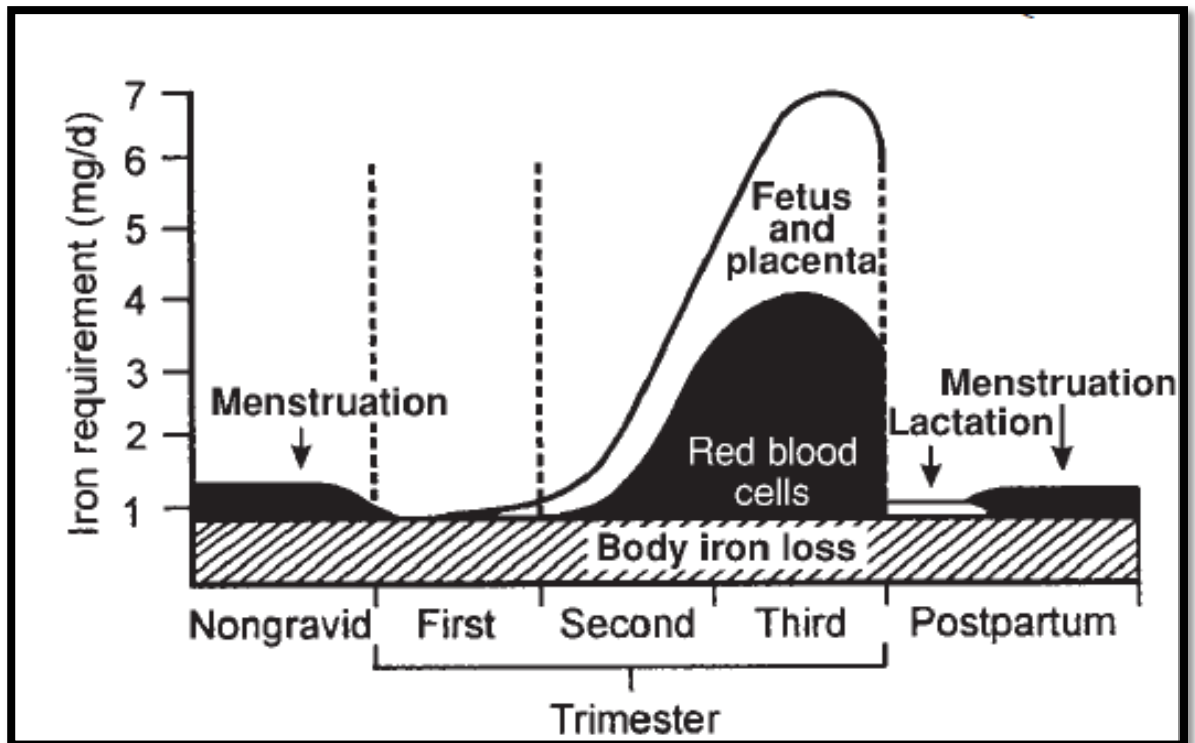
Anemia in pregnancy due to increased iron demand

Anemia in pregnancy is more common pathological state with a prevalence of 57.9 % in India. The Centre for Disease Control and Prevention, 1998 defines anemia in pregnant women who are on

iron supplementation as “hemoglobin (Hb) concentration of less than 11g/dl in the first and third trimesters and less than 10.5 g/dl in the second trimester”. “Post-partum anemia is defined as a hemoglobin concentration of less than 10 g/dl” (MMWR, 1998).

Fall in hemoglobin levels occurs during pregnancy, the fall is mainly due to a disproportionate increase in plasma volume compared with that of red blood cell volume (Hyttén, 1985). The increase in plasma volume is to meet the metabolic needs of an enlarged uterus, hypertrophied vasculature, developing placenta and fetus. Additional elemental iron (about 1000mg) is needed during pregnancy. Of this, 300mg is required for the fetus and placenta, 200mg is lost through the gut, urine and skin and the remaining 500mg compensates for the increase in total volume of erythrocytes (Cunningham et al. 2014).

Figure 11: Iron requirement during pregnancy



Source: Bothwell TH, 2000

Increased iron demands occur after 20 weeks of gestation; the requirement of iron is about 6-7mg/day (Bothwell 2000). The increase in iron requirement is almost doubled when compared with healthy non-pregnant females. This is mainly due to increases in maternal plasma volume. The maternal erythrocyte volume will not be adequate unless iron is supplemented therapeutically, since dietary supplementation and iron stores cannot meet this extra demand (Cunningham et al. 2014). Fetal hematopoiesis is usually not affected, since the placenta transfers iron to the fetal side, even

in conditions of maternal depletion and severe iron deficiency (Cunningham et al. 2014).

Complications of anemia in pregnancy

Anemia in pregnancy has deleterious effects on both the mother and fetus. Maternal complications are based on the degree of anemia; this may range from an asymptomatic state to easy fatigability in mild anemia. In case of severe anemia, the mother can present with dyspnea, edema of dependent parts, shock, cardiac failure, toxemia of pregnancy and preterm labour (Smallwood, 1936). Fetal complications due to severe anemia in pregnancy include high neonatal mortality due to prematurity, still-births, low birth weight and delayed developmental milestones (Cunningham et al. 2014).

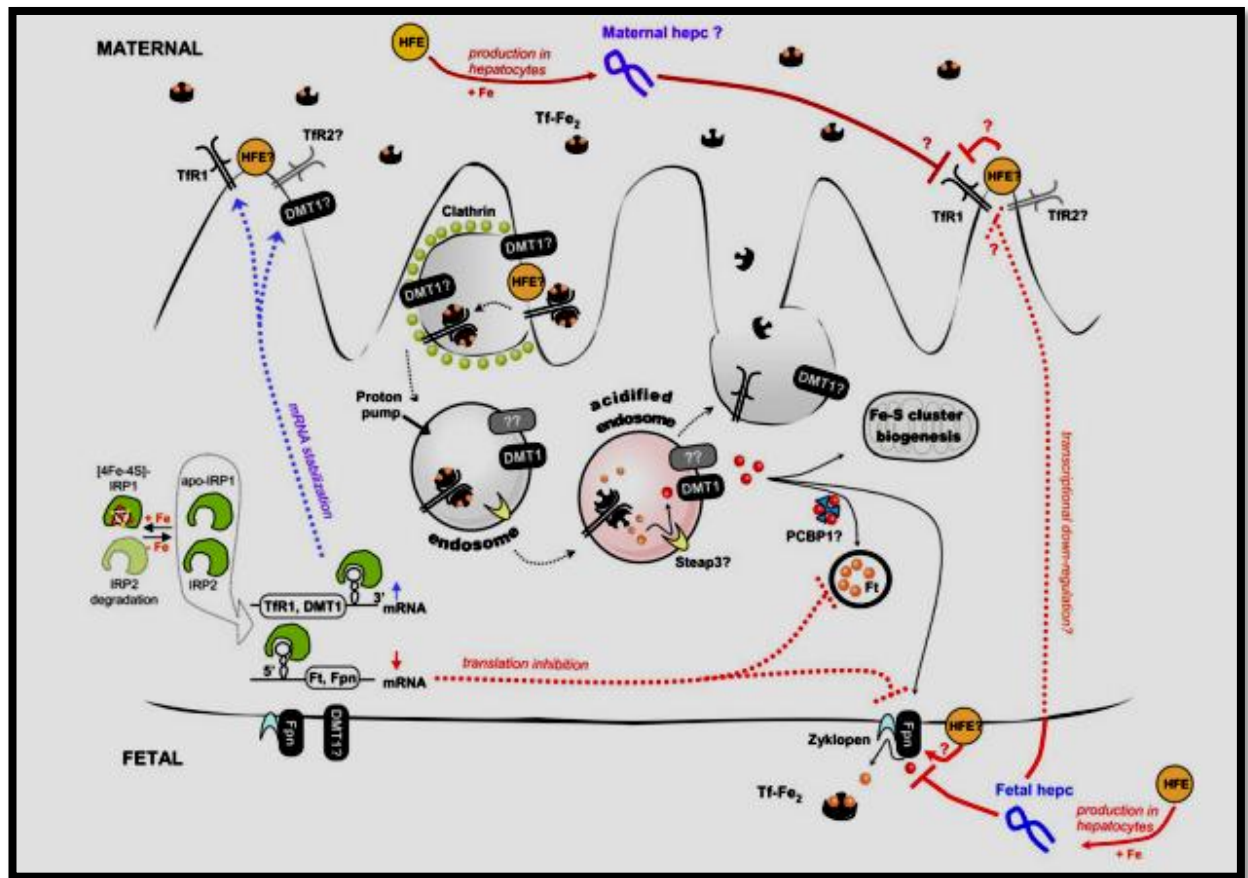
Placenta as a unit for materno-fetal transfer of substances

The placenta is the functional unit mainly involved in maternal-fetal transfer of nutrients and oxygen. The human placenta is hemochorial, even though maternal and fetal blood does not mix, exchange of nutrients and oxygen from mother to fetus occurs (Boyd et al. 1970). Transport across the syncytiotrophoblast is bidirectional.

The decidua is a specially modified endometrium, which favours the function of the placenta (Cunningham et al. 2014). According to the American College of Obstetricians and Gynecologists, pregnancy is established when implantation of the fertilized egg is complete (8-18 days after fertilization) (Cunningham et al. 2014). The formation of the placenta starts as early as the morula stage of the embryo. The trophoectoderm which surrounds the morula differentiates into the trophoblast (Cunningham et al. 2014). The trophoblast plays an important role at the feto-maternal interface, till term of pregnancy. The chorion is a membrane surrounding the chorionic cavity around the embryo (Boyd et al. 1970). It consists of trophoblast and mesenchymal tissue. Chorionic villi are distributed over the entire periphery of the chorionic membrane (Boyd et al. 1970).

The trophoblast is differentiated into an outer layer of syncytiotrophoblast and an inner layer of cytotrophoblast (Cunningham et al. 2014). The cytotrophoblast forms the germinal cells and secretory component of the placenta. The syncytiotrophoblast mainly helps in transport of nutrients and gas at the feto-maternal interface (Cunningham et al. 2014).

Figure 12: Placental transport of iron



Source: Lipinski et al, 2013

The transport of iron across the placenta is unidirectional (Contractor SF et al.1986). Dietary iron, iron stores in the mother and supplemented iron is delivered across placenta to meet the fetal demand. The maternal holo-transferrin binds to transferrin receptor 1 (TfR 1) on the syncytiotrophoblast on the maternal side (McArdle et al. 1984; McArdle et al. 1985;Georgiff et al. 2000). The complex is internalized as endosome. Iron is released from endosome via the DMT1 transporter (Georgiff et al. 2000). Iron transported out of syncytiotrophoblast to reach the fetal circulation via ferroportin on

the basolateral (fetal side) of syncytiotrophoblast (McKie et al. 2000; Abboud et al. 2000). Transferrin has high affinity for ferric (Fe^{3+}) iron. The ferrous iron is converted to ferric iron by a membrane-bound copper oxidase (Danzeisen et al. 2002). Ferric iron released is taken up by fetal transferrin and enters fetal circulation for transport and iron uptake for development of fetus.

Hepcidin levels in pregnancy

In pregnancy, maternal hepcidin levels depend on the maternal iron status and fetal demand. Hepcidin is down-regulated when there is an increased tissue demand for iron. Evidence from animal studies suggests that hepcidin mRNA expression is decreased in maternal liver with iron deficiency and reversed by iron supplementation (Gambling et al. 2009). In the same study they have shown significant negative correlation between fetal hepatic iron levels and hepcidin levels both in mother and fetus. Another study showed levels of maternal hepcidin reduced in rats, as the pregnancy progresses to enable uptake of iron by the fetus; its level returned to normal levels post-partum (Millard et al. 2004).

Transgenic mouse models which over-express hepcidin shows reduced levels of placental transferrin receptor 1 (TfR1), resulting in severe anemia and early death of offspring (Martin et al. 2004).

Another study done on pregnant women during the third trimester and in newborns suggests that maternal hepcidin and iron status in the mother and fetus play important roles in placental transfer of iron (Young et al, 2011). In the same study, it was also reported that heme iron in the diet is absorbed better than non-heme iron; this heme-iron uptake occurs mainly in the third trimester of pregnancy (Young et al, 2011).

Hepcidin is positively correlated with ferritin suggesting there was depletion of iron stores and low hepcidin levels enable the transfer of iron to the fetus. Hepcidin was negatively correlated with erythropoietin, a marker of erythropoiesis in pregnancy (Finkenstedt et al. 2012). Fetal hepcidin levels regulate ferroportin on the basolateral side of syncytiotrophoblast to meet the fetal iron demand (Donovan et al. 2000).

A meta-analysis was recently published by Koenig et al. 2014 on hepcidin and its role in pregnancy. Their searches revealed only 10 human and 6 animal studies on this, showing limited nature of literature in this area. Of the human studies, 6 were cross-sectional designs and 4 were longitudinal studies. This shows the literature in this area is limited and no studies were from Indian population, where the prevalence of anemia in pregnancy is higher. In the review by Koenig et al. (2014), they found maternal serum hepcidin

levels correlated with various hematological indices and markers of iron status from Rehu et al. 2010; Schultze et al. 2008; van Santen et al. 2013. This suggested that well-known biological relationship linking iron indices, hepcidin and erythropoiesis was operational in pregnancy as well. As shown Young et al, 2011 the maternal hepcidin and iron status in the mother and fetus (done by cord blood analysis at the time of delivery) play important roles in placental transfer of iron (Koenig et al. 2014).

In summary, pregnancy is known to be associated with increased iron demand to provide adequate iron for fetal development. Evidence from animal studies also showed that maternal hepcidin levels are decreased during pregnancy. This would be expected to enhance release of iron from maternal stores and increase maternal intestinal iron absorption, which in turn would result in increased transfer of iron to the fetus. However, human studies in this area are limited. In addition, there is no data available from Indian populations, where the prevalence of anemia in pregnancy is high, regarding hepcidin levels during pregnancy. We therefore designed a cross-sectional study to estimate serum hepcidin levels in the first, second and third trimesters of normal pregnancy and in age-mated non-pregnant women. We also aimed to correlate hepcidin levels with hematological parameters and serum ferritin, a marker of iron stores.

THE STUDY

Background of the study

Iron requirements increase during pregnancy. Hepcidin, a peptide hormone produced in the liver is the major regulator of normal iron homeostasis. Evidence from animal studies shows that hepcidin levels are reduced throughout pregnancy and then raised in the post-partum to provide adequate iron for fetal development. Literature in this area on human subjects is very limited.

Objective of the study

The aim and objective of this cross-sectional study was to determine the serum hepcidin levels in subjects without any complication of pregnancies and to correlate it with hematological parameters and ferritin.

MATERIALS

Equipments used

1. Elix and Milli-Q ultrapure water systems (Millipore, USA)
2. Table-top refrigerated centrifuge (MPW R 350, MPW, Poland)
3. Micro plate reader (Model 680, Bio-Rad Laboratories, Inc, UK)

Chemicals and reagents used for estimation of hepcidin

The chemicals and reagents provided by Peninsula Laboratories (Bachem Group, San Carlos, USA) for estimation of hepcidin were:

1. Standard diluent (peptide-free human serum)
2. Lyophilized standard, anti-serum against hepcidin, biotinylated peptide
3. Enzyme immune-assay buffer, streptavidin- horseradish peroxidase (HRP), substrate solution (TMB - 3, 3', 5, 5'-Tetramethylbenzidine solution) and stop solution (2N HCl).

Miscellaneous consumables used

1. Vacutainer blood collection tubes (BD Biosciences, Plymouth, UK).
2. Micro-tubes and centrifuge tubes (15ml) (Tarsons Products Private limited, Kolkata, India).
3. Micro tips (Tarsons Products Private limited, Kolkata, India).

METHODS

The study was approved by the Institutional Review Board (IRB) at Christian Medical College (CMC), Vellore, India (IRB Min. No. 8151 dated 09.01.2013).

SUBJECTS

Women with uncomplicated pregnancy, who attended the antenatal clinic at the Community Health and Development centre (CHAD) Hospital in CMC, Vellore, were recruited.

Inclusion criteria:

Pregnant women with

1. Hemoglobin levels equal to or more than 11 gm/dL in the first and third trimesters and more than 10.5 gm/dL in the second trimester
2. Serum levels of C-reactive protein (CRP) less than 6 mg/dL

Exclusion criteria:

Pregnant women with

1. Hemoglobin levels less than 11 gm/dL in first or third trimesters or less than 10.5 gm/dL in the second trimester.
2. Serum levels of C-reactive protein (CRP) more than 6 mg/dL

3. Any complication of pregnancy
4. Those who were not willing to participate in the study

The pregnant women were recruited into the study on their first antenatal visit. None of them were on iron supplements as far as was ascertainable. Control subjects were non-pregnant women staff of the pre-clinical departments at CMC, Vellore. They were between 25 and 30 years of age.

INFORMED CONSENT

Once pregnant women and control subjects were identified on the basis of the inclusion and exclusion criteria listed above, they were told about the study and invited to participate. They were provided with an information sheet in either Tamil or English, depending on their preference. Once they indicated their willingness to participate, written informed consent was obtained from them. Clinical and socio-demographic information of the subjects was obtained in the proforma used (Appendix 2). Information sheet and informed consent form in English which was used is attached in Appendix 3.

Group	No. of participants	Site of recruitment
Non-pregnant control women	10	Preclinical departments of CMC, Vellore
Pregnant women in their first trimester	10	CHAD hospital (antenatal clinic)
Pregnant women in their second trimester	10	CHAD hospital (antenatal clinic)
Pregnant women in their third trimester	10	CHAD hospital (antenatal clinic)

CALCULATION OF SAMPLE SIZE

The sample size calculation was done based on the study Finkenstedt et al, 2012.

The mean difference of the hepcidin levels between first and third trimester in this study was 6.5 ng/ml. The standard deviation of the hepcidin level was taken as 9 ng/ml. The sample size calculation

was done, using the formula below, with 80% power and 5% level of significance.

Formula:

$$n = \frac{(Z_{\alpha/2} + Z_{1-\beta})^2 2 * s^2}{d^2}$$

$Z_{\alpha/2}$ is the 5% level of significance -1.96

$Z_{1-\beta}$ is 80% power - 0.84

s – Standard deviation (9 ng/ml)

d – Mean difference (6.5ng/ml)

Since the money allocated for a fluid research grant for post graduate dissertation was only Rs. 80,000 for 2 years, it was possible only to estimate only 40 samples with the reagents that were purchased with this amount. Hence, only 40 patients were studied. This included 10 in each trimester and 10 non-pregnant controls.

SAMPLE COLLECTION

Samples were collected from the subjects by venipuncture using BD vacutainer tubes. Approximately 6ml of blood was collected from each subject and control.

Processing of samples

The blood collected was used to estimate hemoglobin and to obtain serum. For the latter, clotted blood in the tubes was centrifuged at 2500 rpm, within 2 hour of blood collection. Serum was separated and divided into 4 aliquots. Additional values of hematological parameters of the subjects (Hematocrit and Mean Corpuscular Volume [MCV]) were obtained from the hospital records of the patients, when available.

Storage of samples

Serum samples were stored at -70°C. They were used for estimation of serum hepcidin, CRP (C-reactive protein) and ferritin.

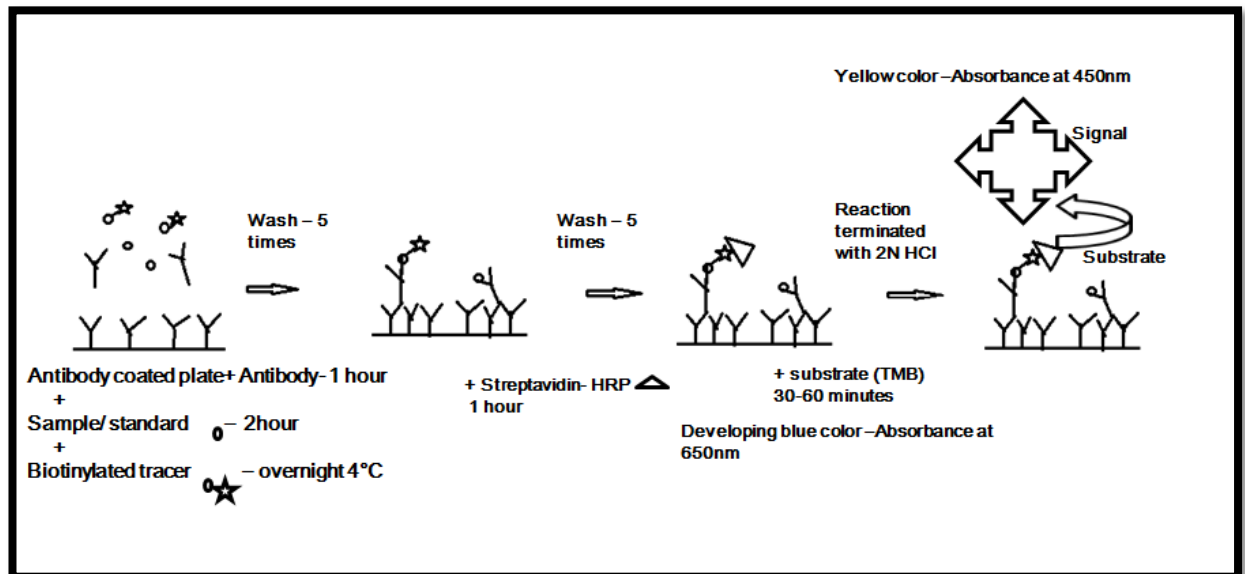
ESTIMATION OF SERUM HEPCIDIN

Reagents for the estimation were purchased from Peninsula Laboratories, (Bachem Group, San Carlos, USA).

Principle of the method

The method used was based on competitive immunoassay. The anti-rabbit antiserum against hepcidin-25 was captured on an antibody (anti-rabbit antibody) coated on a 96-well plate. A fixed concentration of biotinylated tracer (Bt-tracer) and varying concentrations of the standard or the peptide in diluted serum sample competed for binding to the anti-serum. The captured Bt-tracer was subsequently bound by streptavidin-conjugated horseradish peroxidase (SA-HRP), which produced a colored product when substrate was added.

Competitive enzyme immuno assay for serum hepcidin



Components in the kit

1. 96-well coated plate.
2. Enzyme immunoassay buffer (50ml).
3. Standard lyophilized hepcidin peptide (1µg).
4. Standard diluents (8ml) (Peptide-free human serum)
5. Antiserum against hepcidin-25 (Lyophilized powder)
6. Lyophilized powder of biotinylated peptide
7. Streptavidin-HRP -100µl (HRP - horse radish peroxidase)
8. Substrate solution (TMB- 3, 3', 5, 5'-tetramethylbenzidine) (11ml of TMB and hydrogen peroxide [H₂O₂])
9. Stop solution - 2N HCl (hydrochloric acid, 15ml)

As per manufacturer's information, standard diluent, lyophilized standard, antiserum and biotinylated peptide were stored at -20°C. Enzyme immunoassay buffer, streptavidin-HRP, substrate solution and stop solution were stored in a refrigerator (2-4°C). These reagents were stable for one year, under these conditions.

Preparation of the reagents

The unopened kit and reagents were equilibrated to room temperature before preparation of samples and working reagents.

1. Stock standard: To 1µg of lyophilized standard, 1ml of standard diluent was added and mixed, using a vortex mixer.

Standard	ng/ml	Range: 0.02-25 ng/ml
Stock	1000	
S1	25.00	Added 5 µl stock + 195 µl diluent
S2	6.25	Added 40 µl S1 + 120 µl diluent
S3	1.56	Added 40 µl S2 + 120 µl diluent
S4	0.39	Added 40 µl S3 + 120 µl diluent
S5	0.10	Added 40 µl S4+ 120 µl diluent
S6	0.02	Added 40 µl S5+ 120 µl diluent
S0	0.00	120 µl diluent

2. Samples: The 40 samples in the study were diluted 1 in 10 (12 µl of sample + 108 µl of standard diluent)

3. Enzyme immuno assay buffer (EIA buffer): EIA buffer (50ml) was diluted to 1,000 ml with sterile deionized water (18 MOhm) and mixed well.

4. Antiserum: 5ml of EIA buffer was added to the lyophilized antiserum and mixed, using a vortex mixer.

5. Biotinylated-tracer (Bt-tracer): 5ml of EIA buffer was added to the lyophilized powder of Bt- tracer.

6. Streptavidin-HRP: The tube was centrifuged before dilution. It was diluted 1 in 200 with EIA buffer (60 µl of streptavidin-HRP with 12ml of EIA buffer) and mixed, using a vortex mixer.

Layout of 96-well micro titre plate used in the study

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	T1	T1	T9	T9	T17	T17	T25	T25	T33	T33
B	S1	S1	T2	T2	T10	T10	T18	T18	T26	T26	T34	T34
C	S2	S2	T3	T3	T11	T11	T19	T19	T27	T27	T35	T35
D	S3	S3	T4	T4	T12	T12	T20	T20	T28	T28	T36	T36
E	S4	S4	T5	T5	T13	T13	T21	T21	T29	T29	T37	T37
F	S5	S5	T6	T6	T14	T14	T22	T22	T30	T30	T38	T38
G	S6	S6	T7	T7	T15	T15	T23	T23	T31	T31	T39	T39
H	S0	S0	T8	T8	T16	T16	T24	T24	T32	T32	T40	T40

B- Blank; S- standards; T- unknown samples

Procedure

Step 1: Added 25 µl of antiserum to each well and 25 µl of EIA

buffer to the blank well. Incubated at room temperature for 1 hour.

Step 2: Added 50 µl of diluted standard/ sample to each well and

50 µl of diluent in the blank well. Incubated at room temperature for 2 hours.

Step 3: Rehydrated biotinylated-tracer (Bt-tracer) with EIA buffer

and added 25 µl of Bt-tracer per well.

Step 4: Sealed the micro-plate and incubated at 4°C in refrigerator overnight.

Step 5: Re-equilibrated the micro titer plate to room temperature.

Step 6: Washed the immunoplate 5 times with 300 µl of EIA buffer per well.

Step 7: Added 100 µl per well of streptavidin-HRP to all the wells.
Incubated at room temperature for 1 hour.

Step 8: Washed the immunoplate 5 times with 300 µl of EIA buffer per well.

Step 9: Added 100 µl of TMB (substrate solution) per well.
Incubated at room temperature for 30-60 minutes.

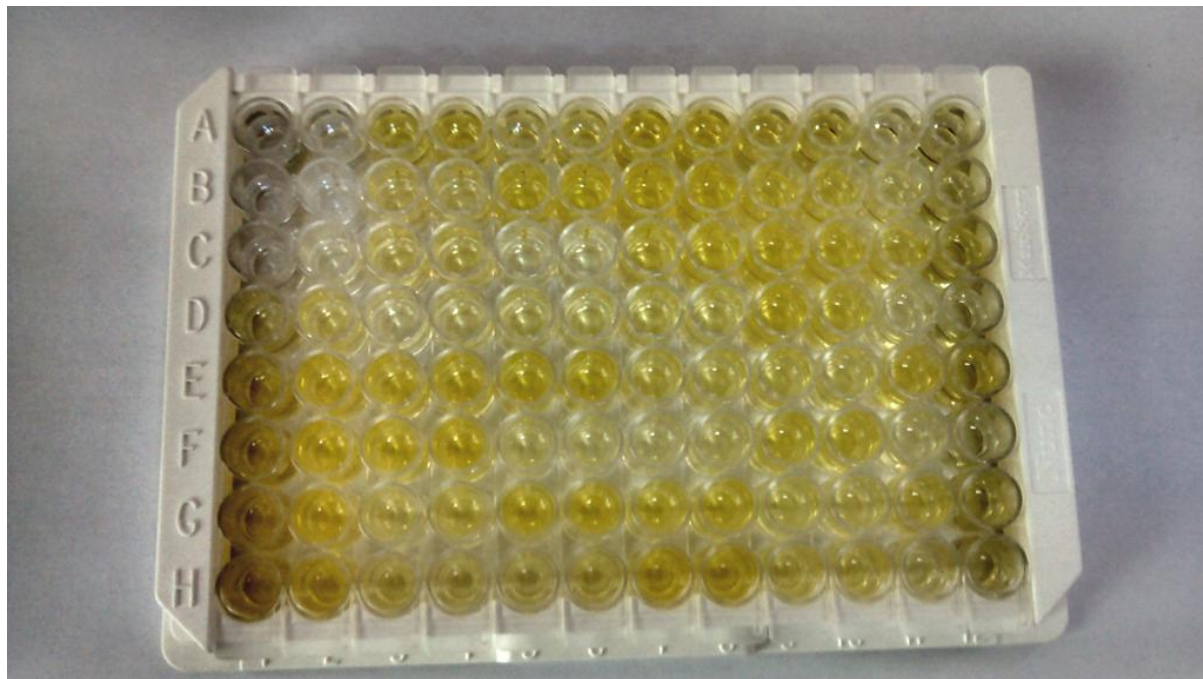
Step 10: Readings were taken at 650nm, during development of blue colour

Step 11: Terminated the reaction with 100 µl of 2N HCl per well.

Step 12: Readings were taken at 450 nm, within 10 minutes of termination

Readings of optical density (OD) were obtained, using the software micro plate manager of the ELISA plate reader. .

Image of microtitre plate after termination of the reaction



A standard curve was plotted on a semi-log scale, using Microsoft Office Excel, 2007. The mean of the OD readings was used for the y axis and the concentrations of the standards (ng/ml) for the x axis. The serum hepcidin levels were calculated using four parameters logistic regression analysis using the following formula.

$$y = \frac{a - d}{1 + (x/c)^b} + d$$

This equation was used to calculate the value of fit and plot **“smooth line of fit”**. The four parameters a (maximum), b (slope), c (IC50, point of inflection), and d (minimum) were adjusted, till the value of fit was satisfied and the fit was good.

$$x = c \left(\frac{y - a}{d - y} \right)^{1/b}$$

This equation was used to calculate the concentrations of samples, expressed in ng/ml.

ESTIMATION OF SERUM C-REACTIVE PROTEIN

CRP levels were estimated in Department of Clinical Microbiology, CMC, Vellore.

Instrument used

Reagents and equipment used for serum CRP analysis were manufactured by Siemens GmbH, Marburg, Germany. The nephelometer used was BN Prospec, Siemens GmbH, Marburg, Germany.

Principle of the method (particle-enhanced nephelometry)

Polystyrene particles coated with monoclonal antibodies specific to human CRP aggregated when mixed with samples containing CRP. A beam of light passed through the aggregates was scattered. The intensity of the scattered light was proportional to the concentration of the relevant protein in the sample. The result was evaluated by comparison with a standard of known concentration.

Estimation of serum CRP for samples from both subjects (about 39 samples) and controls (about 12 samples) was done. Eleven samples were excluded from the study, as the serum CRP levels in these samples were more than 6mg/dl.

ESTIMATION OF SERUM FERRITIN

Estimation of serum ferritin was done in the Department of Clinical Biochemistry, CMC, Vellore.

Analyzer used

Siemens, ADVIA Centaur system Xpi, UK.

Principle of the method (two-site sandwich immunoassay using direct chemiluminescence technology)

Two anti-ferritin antibodies were used in this method. The first antibody was a polyclonal goat anti-ferritin antibody. This antibody was labelled with acridinium ester. The second antibody was a monoclonal mouse anti-ferritin antibody. The second antibody was in the solid phase that was covalently coupled with paramagnetic particles. The amount of ferritin present in the patient's sample was directly proportional the amount of relative light units detected by the system.

DATA ANALYSIS

The Statistical Package for the Social Sciences (SPSS) software package, version 16, was used to analyze the data obtained. Data were subjected to Shapiro-Wilk test to check for normal distribution. Values for serum hepcidin, ferritin and CRP were not normally distributed whereas hematological parameters (hemoglobin, hematocrit and mean corpuscular volume) were normally distributed. Data for hepcidin, ferritin and CRP were analyzed by Kruskal-Wallis test and pair-wise comparison was done using Mann-Whitney test. Hematological parameters were analyzed by Analysis Of Variance (ANOVA).

Correlation analysis was done using Pearson's correlation for normally distributed data and Spearman's rank test for skewed data. Microsoft Office Excel 2007 was used for plotting standard curve fit and regression analysis for hepcidin against gestational age. A p value of <0.05 was considered as statistically significant in all cases.

RESULTS

Characteristics of subjects:

Thirty pregnant women, with uncomplicated pregnancies, and ten non-pregnant women, in the reproductive age group, were the subjects of the study. Ten subjects were recruited in each of the 3 trimesters of pregnancy; those at less than 13 weeks of gestation were considered to be in their first trimester, those between 13 and 26 weeks of gestation were considered to be in the second trimester and those with gestational age greater than 26 weeks were considered to be in the third trimester. Ten non-pregnant females, as described earlier in the methodology section, served as control subjects for the study. The clinical characteristics of the subjects in the different groups are shown in Table 1.

Table 1- Clinical characteristics of subjects

Parameters	Controls	First trimester	Second trimester	Third trimester
Number of Subjects	10	10	10	10
Age (years)	28.10	22.20	22.20	21.70
(\pm SD)	(2.28)	(3.39)	(1.84)	(1.64)
Mean gestational age (weeks ⁺ days)		9 ⁺¹	18 ⁺²	30 ⁺⁶
(\pm SD)		(2 ⁺¹)	(4)	(4)
Parity				
Primi	-	9	9	9
Second pregnancy	-	1	1	1
Height (cm)	160.8	153.4	156	152
(\pm SD)	(2.82)	(3.50)	(6.36)	(6.97)
Weight (kg)	62.6	53.95	57.5	58.3
(\pm SD)	(10.29)	(6.44)	(9.14)	(9.88)

Table 1- Clinical characteristics of subjects (cont'd)

Parameters	Controls	First trimester	Second trimester	Third trimester
Systolic blood pressure (mmHg) (\pm SD)	115.4 (6.04)	110.8 (7.08)	107.1 (12.77)	106.5 (9.14)
Diastolic blood pressure (mmHg) (\pm SD)	72.2 (5.99)	66.6 (9.75)	64 (10.20)	64.5 (12.39)

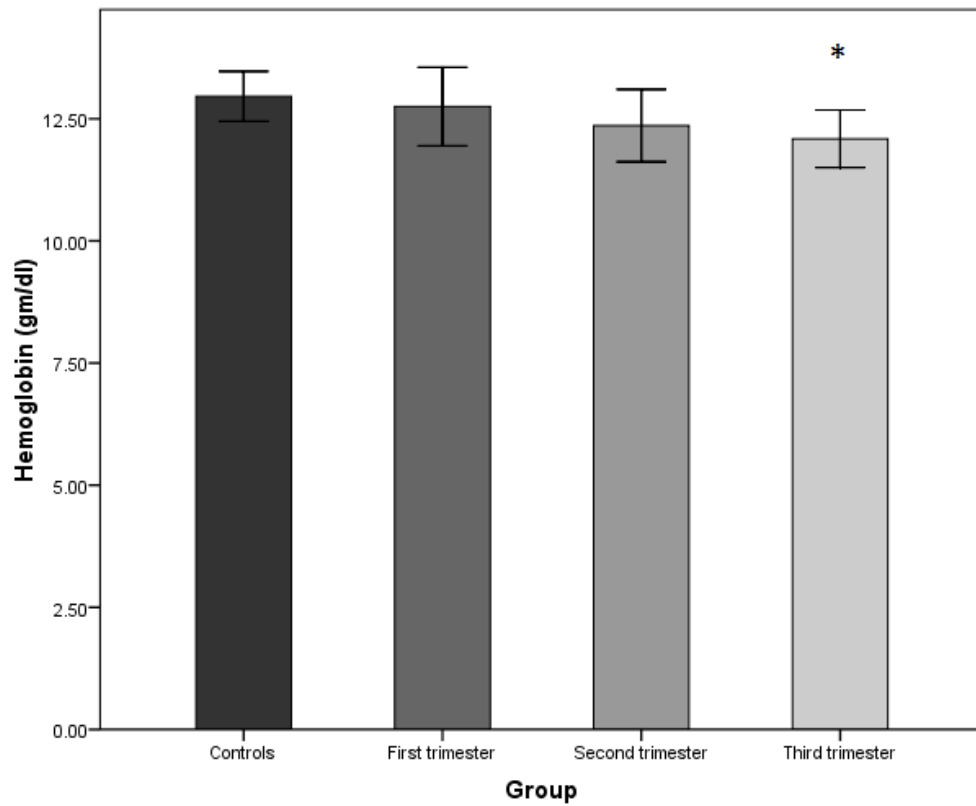
Data were shown as means (\pm SD)

Hematological parameters

The hematological indices measured were hemoglobin, mean corpuscular volume (MCV) and hematocrit. These parameters were found to be normally distributed.

Figures 1, 2 and 3 shows the means of these parameters in the subjects studied.

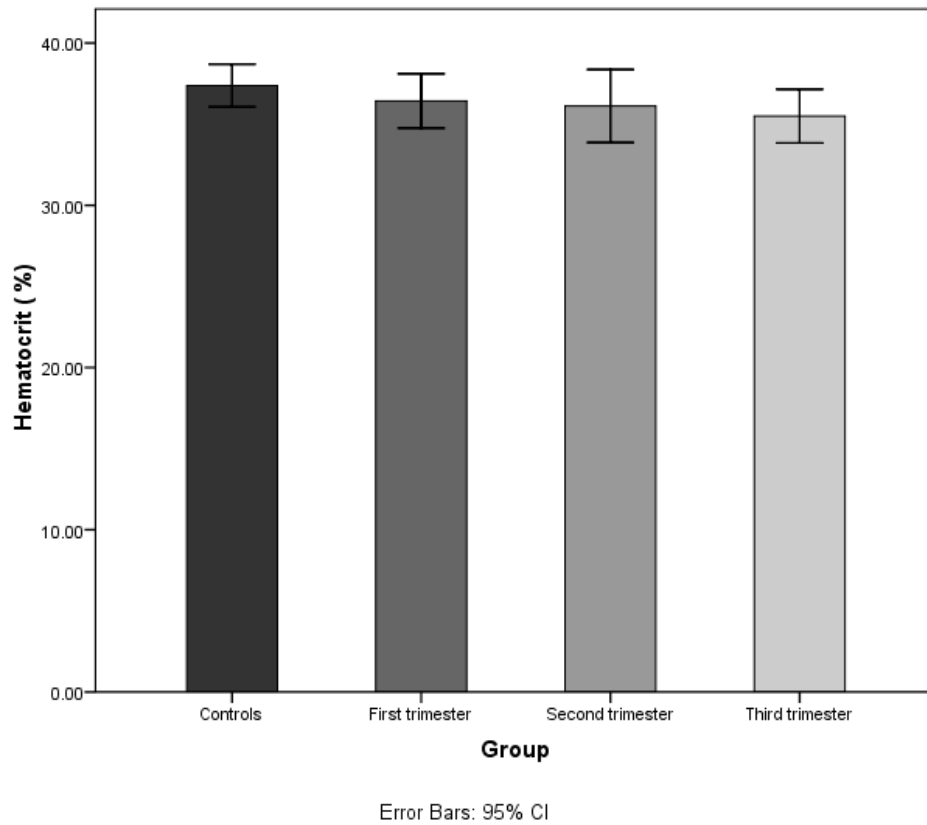
Figure 1. Hemoglobin levels in subjects



Data were analyzed by ANOVA. * $p < 0.05$, when compared with control data.

Hemoglobin levels showed a tendency to decrease with increasing gestational age. Levels in subjects in the third trimester were significantly lower than those of subjects in the control group. Values in the three trimesters did not differ significantly from one another.

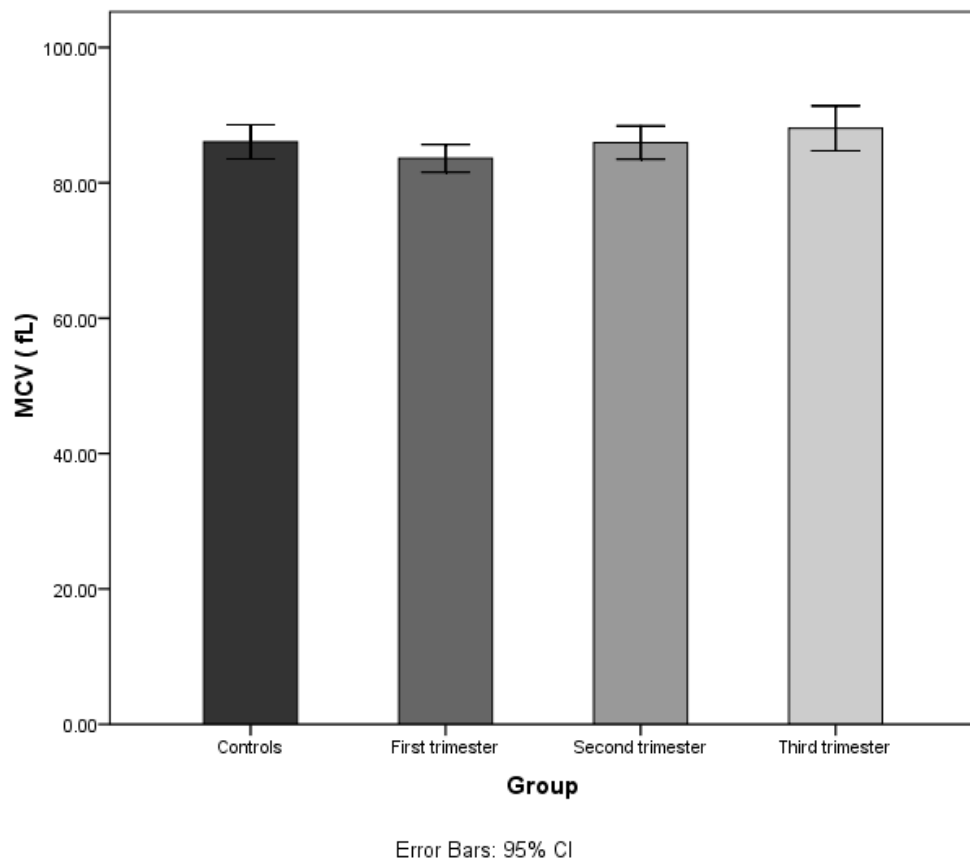
Figure 2. Hematocrit levels in subjects



Data were analyzed by ANOVA.

The hematocrit values in the 3 groups of pregnant subjects were lower than that in the control group; these decreases were, however, not statistically significant. Values in the three trimesters did not differ significantly from one another.

Figure 3. Mean corpuscular volumes in subjects



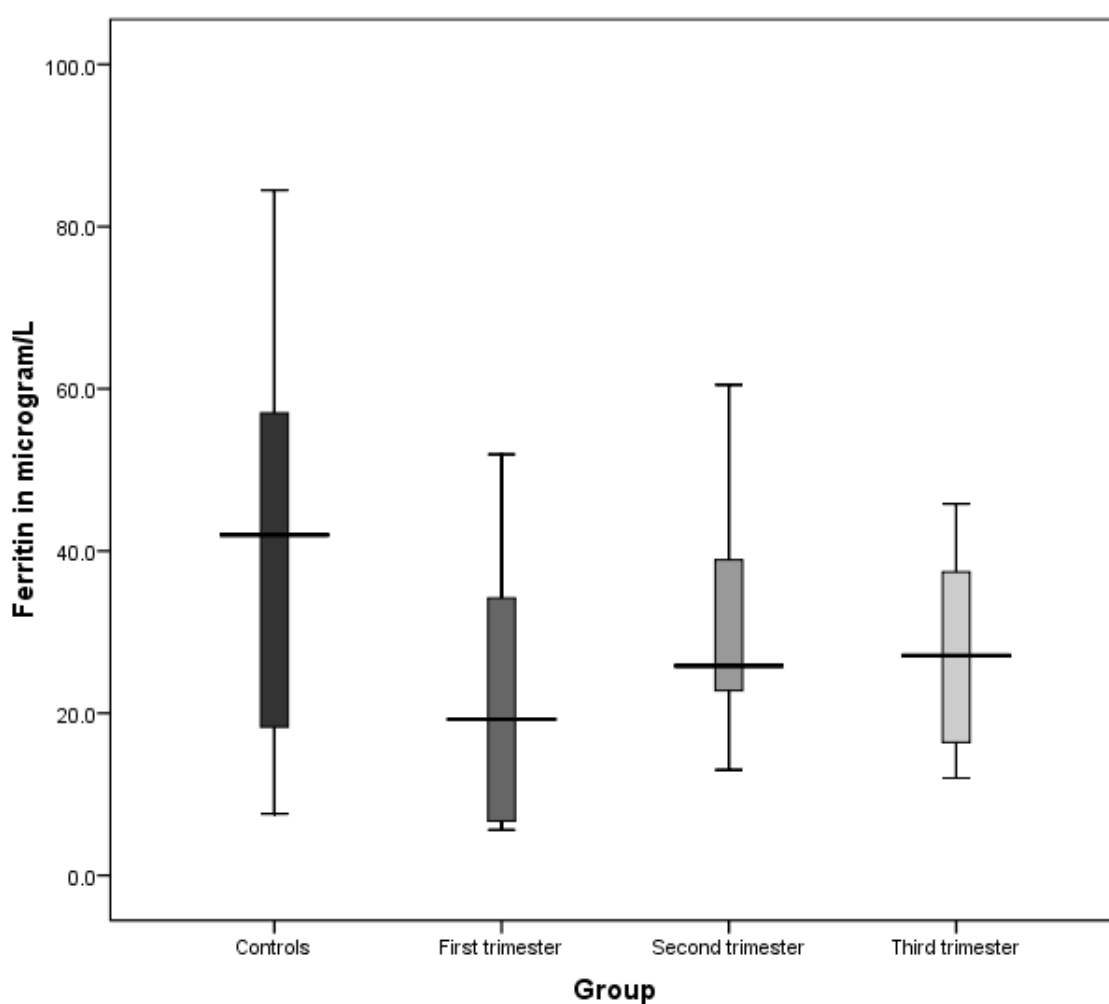
Data were analyzed by ANOVA.

The MCV in the groups studied were not significantly different from one another.

Serum ferritin

Serum ferritin was measured as a marker of iron status. The values of this parameter were found to have a skewed distribution. The median values of ferritin in the 4 groups are shown in Figure 4.

Figure 4: Ferritin levels in subjects



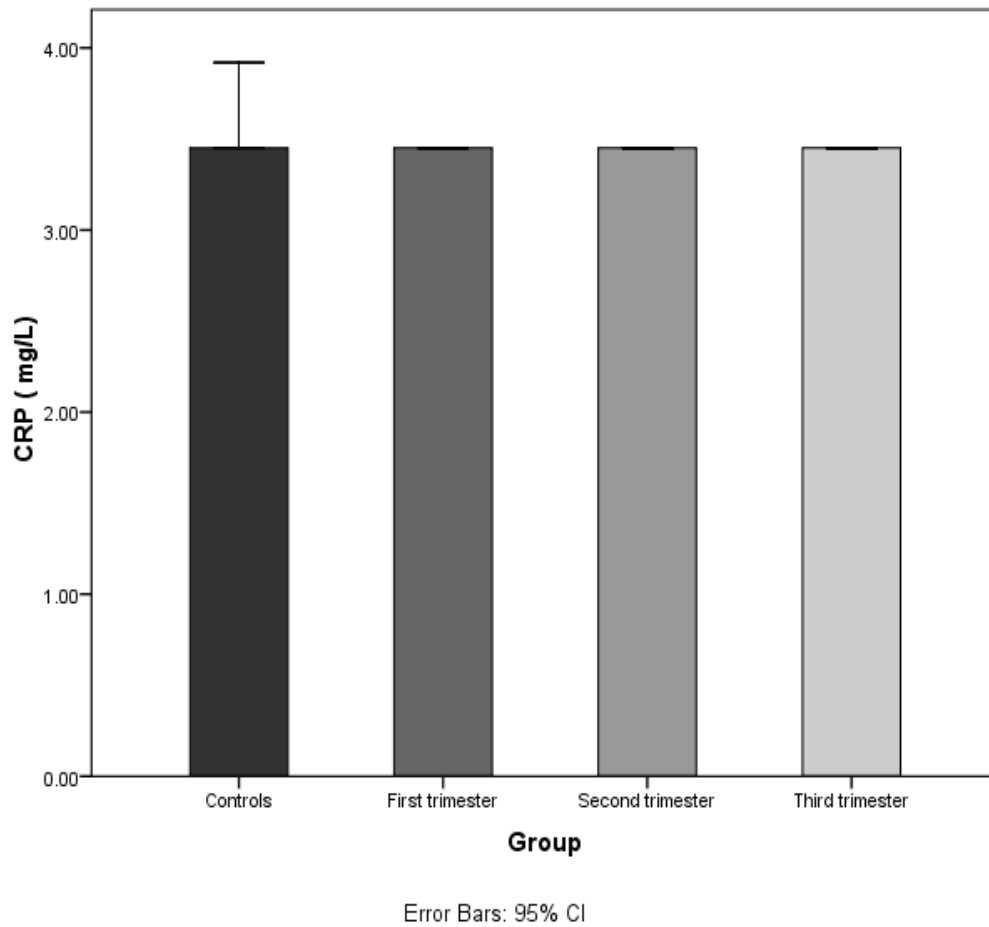
Data are shown as box and whisker plots, showing quartiles and medians. Analysis of data was done using Kruskal-Wallis test.

Serum ferritin levels were lower in the pregnant subjects than in the control group. These decreases were, however, not statistically significant. Values in the three trimesters did not differ significantly from one another.

Serum C-reactive protein

Serum C-reactive protein (CRP), a marker of inflammation, was estimated in the subjects of the study. Only subjects with CRP less than 6 mg/L were included in the study. The values obtained for serum CRP were found to have a skewed distribution.

Figure 5. Serum CRP levels in subjects



Data are shown as medians. The range of values for each group is as follows: Controls (3.45 - 4.85); first trimester (3.45 - 5.71); second trimester (3.45 - 5.59); third trimester (3.45 - 3.45). Analysis of data was carried out using Kruskal-Wallis test.

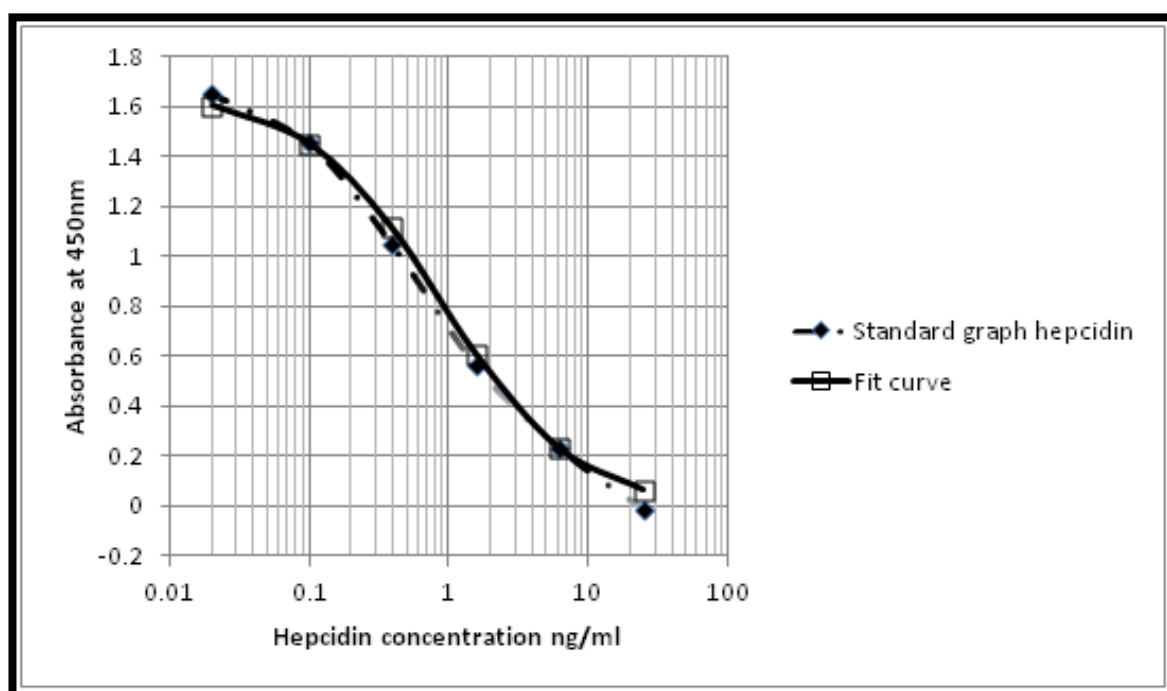
There were no significant differences in the values for CRP among the groups studied.

Serum hepcidin levels

Serum hepcidin was measured by ELISA, using a commercially available kit (Bachem Group, San Carlos, USA). A standard curve was plotted on a semi-log scale, using Microsoft Office Excel 2007. The concentrations of the standards (ng/ml) were used for the x axis; the mean of the optical density (OD) readings obtained for each standard was used for the y axis.

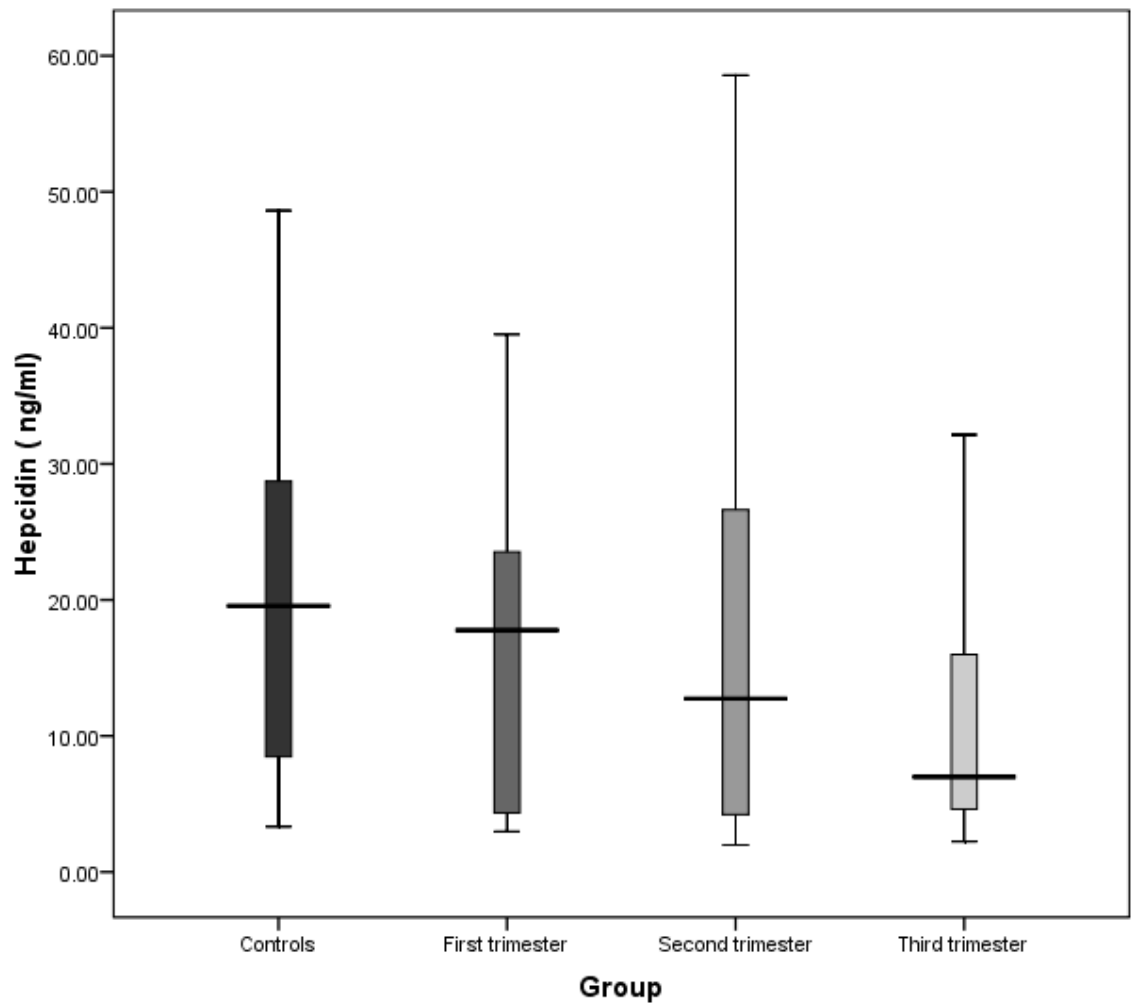
The standard curve generated is shown in Figure 6. Concentrations of hepcidin levels in each sample were calculated from this curve.

Figure 6: Standard curve for hepcidin



Values for serum hepcidin levels in the patients' samples were not normally distributed. The median values of hepcidin in the groups are shown in Figure 7.

Figure 7: Serum hepcidin levels in subjects

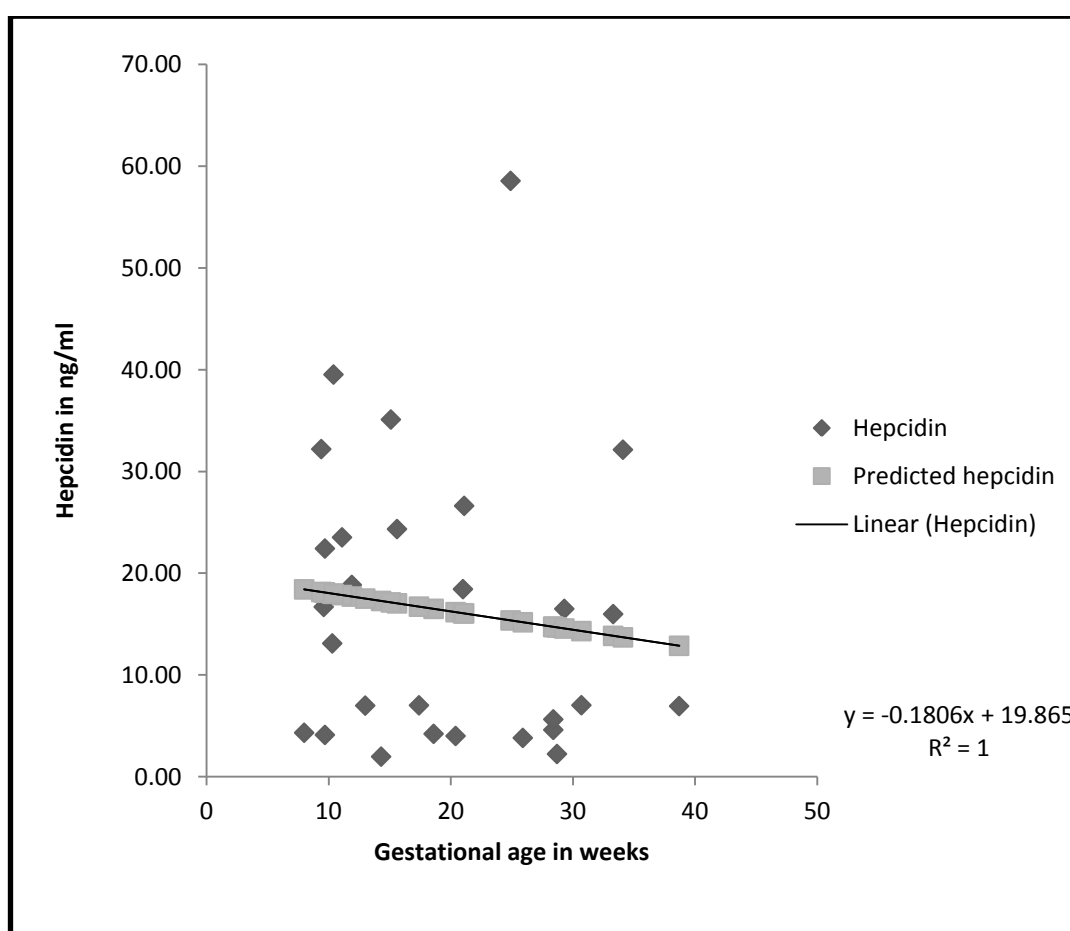


Data are shown as box and whisker plots, showing quartiles and medians. Analysis of data was done using Kruskal-Wallis test.

Median hepcidin levels in pregnant subjects were found to be lower than in control subjects. They showed a tendency to decrease with increasing gestational age. The decreases were, however, not statistically significant.

Serum hepcidin levels in pregnant subjects were plotted against their gestational ages by regression analysis with Microsoft Office Excel 2007.

Figure 8: Serum hepcidin levels versus gestational age



Serum hepcidin levels showed a trend to decrease with increases in gestational age

Table 2: Correlation analysis for normally distributed variables

Correlation	r-value	p-value
All subjects (non-pregnant and pregnant subjects)		
Hemoglobin vs hematocrit	0.906	0.000
Non-pregnant subjects		
Hemoglobin vs hematocrit	0.906	0.000
Pregnant subjects		
Hemoglobin vs hematocrit	0.906	0.000
First trimester		
Hemoglobin vs hematocrit	0.910	0.000
Second trimester		
Hemoglobin vs hematocrit	0.910	0.000
Third trimester		
Hemoglobin vs hematocrit	0.905	0.000

Data were analyzed by Pearson correlation coefficient.

Hemoglobin correlated positively with hematocrit values in non-pregnant subjects, in pregnant women in all three trimesters and also in the total combined group of pregnant and non-pregnant subjects.

Table 3: Correlation analysis for data with skewed distributions

Correlation	r-value	p-value
All subjects (non-pregnant and pregnant subjects)		
Ferritin		
vs hemoglobin	0.384*	0.015
vs hematocrit	0.282	0.077
vs MCV	0.305	0.056
Hepcidin vs MCV	-0.300	0.060
Non-pregnant subjects		
Ferritin vs MCV	0.636*	0.048
All pregnant subjects		
Hemoglobin vs gestational age	-0.353*	0.026
First trimester		
Ferritin vs hemoglobin	0.723*	0.018
vs hematocrit	0.624	0.054
Hepcidin vs MCV	-0.673*	0.033
Third trimester		
Ferritin vs hemoglobin	0.646*	0.043

Data were analyzed by Spearman's correlation coefficient.

*p < 0.05

Various significant correlations were seen among the data with skewed distribution. When the total data, from both non-pregnant and pregnant subjects, were analysed, a significant positive correlation was found between ferritin and hemoglobin; there was also a tendency for a similar relationship between ferritin and haematocrit and MCV. In this group, there was also a tendency for serum hepcidin and MCV to be negatively correlated.

When non-pregnant subjects were considered alone, ferritin levels and MCV correlated positively with one another. Among pregnant women, hemoglobin levels negatively correlated with gestational age. Positive correlations were found between ferritin and hemoglobin in the first and third trimesters and with haematocrit in the first trimester. Hepcidin and MCV levels correlated negatively in the first trimester.

Summary of findings

1. Hemoglobin levels were lower in pregnant women than in non-pregnant ones, with the decrease being significantly lower in women in the third trimester.
2. Values for hematocrit and mean corpuscular volume in the 3 groups of pregnant women tended to be lower than those in the control group, but not significantly so.
3. Serum ferritin levels tended to be lower in the pregnant women than in non-pregnant women, but not significantly so.
4. Serum hepcidin levels were found to fall with increasing gestational age, but were not significantly different in the groups studied.
5. Hemoglobin correlated positively with hematocrit and ferritin levels in both non-pregnant and pregnant women.
6. Hepcidin correlated negatively with mean corpuscular volume in the first trimester; there was a tendency to the same correlation in the combined group of non-pregnant and pregnant women as well.

DISCUSSION

Knowledge about mechanisms involved in iron homeostasis has progressed tremendously in the last 2 decades. The discovery of proteins involved in these processes has helped to better understand disorders related to iron metabolism (Andrews and Schmidt 2007).

Hepcidin is the major regulatory hormone that maintains systemic iron levels (Murray et al. 2012). It is regulated by many processes; erythropoiesis and hypoxia cause it to be down-regulated (Nicolas et al. 2002), while inflammation and iron overload cause it to be up-regulated (Nemeth et al. 2004; Pigeon et al. 2001).

Pregnancy is associated with apparently decreased maternal serum iron levels; this is due to hemodilution and also due to increased demands for iron by the fetus (Cunningham et al. 2014). The increase in plasma volume in pregnancy, which is essential to meet the metabolic needs of an enlarged uterus, hypertrophied vasculature, developing placenta and the fetus, is responsible for hemodilution.

In pregnancy, an increased rate of erythropoiesis is required for fetal development and this may lead to down-regulation of hepcidin. About 1000 mg of elemental iron is needed during pregnancy; this

exceeds normal iron stores in females (Longo et al. 2011). It is expected that maternal hepcidin levels would be down-regulated in pregnancy to provide more iron to the placenta, for transfer to the fetus.

Iron is transported across the placenta through ferroportin, which is located on the baso-lateral side of the syncytiotrophoblast (Donovan et al. 2000). Ferroportin at this site is regulated by fetal hepcidin (Donovan et al. 2000). Both maternal and fetal hepcidin levels determine the transfer of iron across the placenta. A previous study has shown that cord blood hepcidin levels did not correlate with markers of maternal iron status, but correlated well with markers of iron status in the fetus (Rehu et al. 2010). Another study done on pregnant women, in their third trimester and in newborns, suggests that maternal hepcidin and iron status in the mother and fetus play important roles in placental transfer of iron (Young et al, 2011).

Hepcidin has been shown to be down-regulated as pregnancy progresses, in keeping with increased iron demands (Finkenstedt et al. 2012) . In the same study, the authors have reported a negative correlation between serum levels of hepcidin and erythropoietin. A study by van Santen et al (2013) has shown that hepcidin levels were decreased in the second and third trimesters and increased in

the post-partum period. This has also been reported by Gyarmati et al. (2011); however, the increases seen did not correlate with serum iron and ferritin levels. Studies on rats have also shown that maternal hepcidin levels fell as pregnancy progressed; these returned to normal levels in the post-partum period (Millard et al. 2004).

In the present cross-sectional study, serum hepcidin levels showed a trend to decrease with increasing gestational age. The falls seen were, however, not statistically significant. The non-significant nature of the change may be accounted for by the small sample size in the study. Decreases in serum hepcidin levels with progression of pregnancy have also been reported by others, with lowest levels reported in the third trimester (Finkenstedt et al. 2012; van Santen et al 2013; Dao et al. 2013). Hemo-dilution may be a factor that contributes to such a decrease. Another factor is likely to be increased iron demands by the fetus. With increasing gestational age, fetal requirements for iron increase. Hepcidin levels would need to be down-regulated to meet such increased requirements (Blackburn, 2003). Animal studies have also documented decreased hepcidin expression with progression of pregnancy and increases post-partum (Millard et al. 2004, Cornock et al, 2013). However, it is not known what specifically regulates hepcidin levels in pregnancy (Koenig et al, 2014).

Estimation of serum hepcidin levels have been done by different methods. These include mass spectrometry, cation exchange chromatography and enzyme linked immunosorbent assay for different studies (Finkenstedt et al. 2012; van Santen et al. 2013; Dao et al. 2013). In the present study a commercially available ELISA kit was used for estimation of serum hepcidin levels. The median hepcidin levels in the present study are similar to those reported by Finkenstedt et al. (2012) in pregnant women, even though the methods of estimations differ. It is often difficult to make direct comparison of serum hepcidin levels when estimations have been done by different methods (Koenig et al. 2014). Comparison of hepcidin levels would be easier if reference interval at different stages of pregnancy was known (Koenig et al. 2014).

Koenig et al. (2014) have carried out a meta-analysis of publications that have studied hepcidin in pregnancy. Their searches revealed only 10 human and 6 animal studies on this, showing limited nature of literature in this area. Of the human studies, 6 were cross-sectional designs and 4 were longitudinal studies. None were done on Indian populations. Sample size in these studies varied from as little as 19 to as many as 191 and included those done at various time points during pregnancy and also in the post-partum period. The methods used for estimation included mass spectrometry and ELISA. The authors concluded that it is difficult

to make direct comparisons among the various estimations done because of a lack of standardization of the methods used. Reference ranges for hepcidin levels in pregnancy are not available and need to be established (Koenig et al, 2014).

Maternal serum hepcidin levels have been shown to correlate with various hematological indices and markers of iron status (Rehu et al. 2010; Schultze et al. 2008; van Santen et al. 2013). This suggested that the well-known biological relationship linking iron indices, hepcidin and erythropoiesis was operational in pregnancy as well. The present study showed a correlation only between serum hepcidin and MCV, but not the other hematological parameters and indices of iron status. This may be the reflection of small sample size in the study. In the post-partum period, hepcidin levels have been shown to rise; however at this point, it did not correlate with markers of iron status (Rehu et al. 2010; Schultze et al. 2008; van Santen et al. 2013).

Inflammation is known to cause up-regulation of hepcidin transcription (Nemeth et al. 2004). Because of this, it was necessary to rule out the presence of inflammation in the subjects. In order to do this, only subjects with serum C-reactive protein levels less than 6 mg/L were recruited for the present study. Hence, it is certain

that inflammatory factors did not influence serum levels of hepcidin in these patients.

Serum ferritin levels in pregnancy are affected by factors such as hemodilution and iron supplementation (Burtis et al. 2012). In pregnancy, it has been shown that transferrin saturation or a sTfR/ferritin ratio are better indicators of iron status than serum ferritin levels by themselves (Burtis et al. 2012). Evidence from an earlier study has shown positive correlations between serum levels of hepcidin and ferritin (Finkenstedt et al. 2012), suggesting that maternal iron stores get depleted during pregnancy, thus causing hepcidin to be down-regulated. A longitudinal study showed that serum hepcidin levels positively correlated with serum ferritin levels and transferrin saturation and negatively with soluble transferrin receptor throughout pregnancy; the correlation with ferritin was not seen 24 hours post-partum (van Santen et al. 2013). Such a correlation was not seen in the present study, once again, probably due to the cross-sectional design of the study and the small sample size.

In studies done on normal healthy, non-anemic pregnant women on iron supplementation, parameters of iron status, such as serum iron, transferrin saturation and ferritin levels, were found to decrease with the progression of pregnancy and increased after

delivery (Choi et al. 2000). In the present study, ferritin levels were lower in pregnant subjects, when compared with control subjects. This may be explained by the fact that maternal iron stores get depleted in pregnancy. However, no correlation was found between serum levels of hepcidin and ferritin in the groups studied. Once again, the small sample size of the present study may account for this. Larger numbers of subjects will need to be studied to further study these relationships.

A recent study in Indian pregnant women showed no correlation between ferritin and red cell indices (Tiwari et al. 2013), unlike the one by Byg et al (2000). In the present study, ferritin levels in pregnant women correlated with hemoglobin levels; this observation is in keeping with the findings of Byg et al (2000). One of the reasons for the lack of agreement with the findings of Tiwari et al may be the fact that none of the subjects of this study were anemic, as diagnosed by WHO criteria for anemia in pregnant women, while in the study by Tiwari et al, nearly 50% of the women had anemia. In the present study, ferritin also tended to be positively correlated with mean corpuscular volume, again in keeping with the findings of Byg et al (2000). In the present study, hemoglobin levels significantly decreased in third trimester when compared with controls. The finding that hemoglobin decreased with increasing

gestational age is likely to be a result of hemodilution with significantly lower levels in the third trimester.

The cross- sectional nature of the present study is a limitation. It would be best to carry out longitudinal studies to determine hepcidin levels in the course of pregnancy, as has been done by Simavli et al (2014). These authors have shown that elevated serum hepcidin levels were associated with adverse outcomes of pregnancy. It was not found that it was not possible to follow the patients in the study up to delivery to determine the outcome of pregnancy.

Conclusion

Serum hepcidin levels tended to decrease with increasing gestational age, but the decreases seen were not statistically significant. Since the sample size in this study was small, further studies in this area are warranted to confirm these findings and to further study the complex biological relationships between hepcidin and iron metabolism in pregnancy.

Limitations of the study

1. The sample size for the study was small. A larger number of patients will need to be studied to elucidate more clearly the relationship between hepcidin and iron-related parameters in pregnant women.
2. It was not possible to assess the outcomes of pregnancy with regard to the status of the babies born. There were difficulties in obtaining information on gestational age at delivery of the subjects in the study (to determine whether the babies were born preterm, at term or post term) and weight of the babies (normal/low birth weight/ high birth weight). These limitations were due to an inability to trace the all the subjects at a later date to see if they had delivered at CHAD hospital.
3. It was not possible to carry out cord blood analysis on the babies born to the subjects of the study. Such analyses would be useful to shed light on the complex relationships involved in handling of iron in pregnancy.
4. It was difficult to obtain reliable information from the pregnant subjects about iron supplementation. Women in the first trimester said they had been given some tablets from village health nurses

(VHN). These were assumed to be tablets of iron and folic acid, but there was no way to verify this. Women in the second and third trimesters stated that they had taken some such tablets earlier in their pregnancy but had not been taking them in the recent past. However, none of these facts could be verified for certain.

5. It was not possible to study other relevant parameters such as transferrin saturation and serum levels of soluble transferrin receptor, due to financial constraints.

6. A longitudinal study to determine serum levels of hepcidin over in the course of pregnancy would have been ideal. Such a study would have allowed for assessment of relationships of parameters of interest with outcomes of pregnancy.

Future directions

Longitudinal studies to on iron-related parameters in the mother in the course of uncomplicated pregnancies, along with cord blood analysis, will be useful for a better understanding of iron metabolism in the mother and the fetus. Studies on iron-related proteins in the placenta in these patients would also be useful in this regard.

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APPENDIX

Appendix1: Institutional Review Board Approval for the study

OFFICE OF THE VICE PRINCIPAL (RESEARCH)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002

Ref: FG/8151/01/2013

11th February, 2013

The Treasurer
Christian Medical College,
Vellore.



Dear Mr. Denzil,

Sub: **FLUID Research grant project NEW PROPOSAL:**
Serum hepcidin levels in pregnancy.
Ms. P. Gnanapraha, PG Demonstrator, Biochemistry, Dr. Molly Jacob,
Dr. Joe Varghese, Biochemistry, Dr. Jasmine Prasad, Community Health,
Dr. VisalakshiJeyaseelan, Biostatistics.

Ref: IRB Min. No. 8151 dated 09.01.2013

The Institutional Review Board at its meeting held on 09th January, 2013 vide IRB Min. No. **8151** accepted the project for 2 Year at a total sanction Rs. 80,000/- (Rupees Eighty Thousand Only). A sum of Rs 40,000/- will be sanctioned for 12 months after receipt of the revised proposal, subsequent installment of 40,000/- each will be released at the end of the first year following the receipt of the progress report. Kindly arrange to transfer the sanctioned amount to a separate account to be operated by Ms. P. Gnanapraha and Dr. Molly Jacob.

Thank you.
Yours sincerely,


Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
ADDRESS AND NAME AND (Encl) FRACP(Encl) FRCP(Encl)
Secretary (Ethics Committee)
Institutional Review Board

CC: Ms. P. Gnanapraha, Department of Biochemistry, CMC.
Dr. Molly Jacob, Department of Biochemistry, CMC.
File

22.2.2013. Ref. Ms. P. Gnanapraha - Biochemistry (8151)

Appendix 2: Proforma for the study

PATIENT DETAILS PROFORMA

1. NAME: ; HOSP NO: DATE:

2. AGE:

3. NAME OF THE SPOUSE:

4. FAMILY INCOME:

5. ADDRESS:

6. HOSPITAL NO:

7. PHONE NO:

8. SOCIO DEMOGRAPHIC DATA:

OCCUPATION: SELF-

SPOUSE-

EDUCATION: SELF-

SPOUSE-

TYPE OF HOUSE-

TYPE OF FAMILY-

11. OBSTETRIC STATUS: (GPLA):

EDD-

TRIMESTER: FIRST/ SECOND/ THIRD

GESTATIONAL AGE IN WEEKS-

IRON FOLIC ACID STARTED ON-

12. MENSTRUAL HISTORY:

AGE AT MENARCHE-

LMP (FIRST DAY OF LAST MENSTRUAL CYCLE):

MENSTRUAL CYCLE: REGULAR/ IRREGULAR; EVERY DAYS.

H/O OCP-

DATE OF CONFIRMATION OF PREGNANCY-

9. HISTORY OF PREVIOUS PREGNANCY:

ORDER	YEAR OF DELIVERY	FT/ PT	VAGINAL/C-SECTION	LENGTH OOF ABOUT	MALE/ FEMALE CHILD	PLACE OF DELIVERY

FT- FULL TERM; PT- PREMATURE; ORDER- ORDER OF PREGNANCY.

H/O MISCARRIAGE, ABORTION, MOLAR, ECTOPIC PREGNACY

H/O TREATMENT FOR INFERTILITY
H/O ANY COMPLICATIONS IN PREVIOUS PREGNANCY: ANEMIA, HEPATITIS,
GESTATIONAL DIABETES, HYPERTENSION and SEIZURE.
H/O BLOOD TRANSFUSION-
H/O RH-INCOMPATABILITY-
H/O BREAST FEEDING- HOW LONG-
H/O CONTRACEPTIVE METHODS FOR SPACING-

11. MEDICAL HISTORY:

HEART DISEASE- DIABETES- HYPERTENSION-
RECURRENT UTI- EPILEPSY/SEIZURE ON TREATMENT-
LIVER DISEASE: HEPATITIS- ASTHMA-
HYPERTHYROID/HYPOTHYROID- BLEEDING DISORDERS-
H/O DRUGS FOR ANY ILLNESS-
H/O DRUG ALLERGY-
H/O SMOKING, ALCOHOLISM-
PREVIOUS HISTORY OF ANY SURGERY-

12. PHYSICAL EXAMINATION:

HT- WT- BMI-
PULSE- BP-
PALLOR-
ABDOMINAL EXAMINATION FINDINGS-
FETAL HEART SOUND-
VAGINAL EXAMINATION FINDINGS-

13. CLINICAL INVESTIGATION:

TRIMESTER: FIRST/ SECOND/ THIRD
GESTATIONAL AGE IN WEEKS-

FIRST VISIT:

PCV-
HBsAG-
HIV-
VDRL-
BLOOD GROUP Rh ANTIGEN-
URINE: ALBUMIN- SUGAR-
HB-
CRP-
SERUM IRON-
SERUM FERRITIN-
SERUM HEPcidIN-

Appendix 3: Patient information sheet and consent forms

Title of study: Serum hepcidin levels in pregnancy

Investigators: Gnanapraba P, Joe Varghese, Jasmine Prasad,
Visalakshi Jeyaseelan, Molly Jacob

Departments: Department of Biochemistry, Christian Medical College,
Bagayam, Vellore; CHAD Hospital, Christian Medical College, Bagayam,
Vellore

The Department of Biochemistry, Christian Medical College (CMC), Vellore in association with Community Health and Development (CHAD) Hospital, CMC, Vellore is carrying out a study to try to understand why pregnant women often become anemic. Often anemia occurs in pregnancy because the body does not have adequate amounts of iron in the body. We have permission to conduct a study to measure various substances in blood that are associated with iron and the development of anemia. For this, we would like to request for a sample of blood (10 ml) from you. Taking this sample of blood from you will not cause you any foreseeable harm. The information that we get from these measurements will help us understand better the mechanisms involved in development of anemia in pregnancy. In turn, we hope that this will help in development of more effective ways to deal with the problem. You will not be directly benefitted by this study but your participation will help to acquire advance knowledge in this area of medicine. The blood sample we take will be used only for research purposes. If any sample remains after we complete this study, we would

like to request you for permission to store the sample and to use it for future studies that we may conduct on anemia and pregnancy. All information that we collect from you will be kept strictly confidential. If you are not willing to participate in the study you are free to say so. Whether you are part of the study or not will not affect your treatment in CHAD hospital.

Dr.Gnanapraba.P; Contact no: 09444091561.

Dr. Molly Jacob: 04162284267

Dr.Jasmine Prasad: 04162284207

INFORMED CONSENT FORM FOR SAMPLE OF BLOOD.

Dr.Gnanapraba P. has explained to me the details of the study proposed and what part I have in it. I have understood what she has told me. I am willing to participate in the study. I give consent for 10 ml of blood to be collected from me. I understand that this will not affect my health in any foreseeable manner. The blood sample collected will be used only for research purposes. If there is any sample remaining after this study is completed, I give permission for the sample to be stored and used for any related future studies.

Signature/thumb impression of the subject

Signature of investigator

Date